Non fitting based FRET–FLIM analysis approaches applied to quantify protein–protein interactions in live cells

Sergi Padilla-Parra · Nicolas Auduge ·
Maite Coppey-Moisan · Marc Tramier

Received: 3 March 2011 / Accepted: 26 April 2011 / Published online: 17 May 2011
© International Union for Pure and Applied Biophysics (IUPAB) and Springer 2011

Abstract New imaging methodologies in quantitative fluorescence microscopy and nanoscopy have been developed in the last few years and are beginning to be extensively applied to biological problems, such as the localization and quantification of protein interactions. Fluorescence resonance energy transfer (FRET) detected by fluorescence lifetime imaging microscopy (FLIM) is currently employed not only in biophysics or chemistry but also in bio-medicine, thanks to new advancements in technology and also new developments in data treatment.

FRET–FLIM can be a very useful tool to ascertain protein interactions occurring in single living cells. In this review, we stress the importance of increasing the acquisition speed when working in vivo employing Time-Domain FLIM. The development of the new mathematical-based non-fitting methods allows the determining of the fraction of interacting donor without the requirement of high count statistics, and thus allows the performing of high speed acquisitions in FRET–FLIM to still be quantitative.

Keywords Quantitative fluorescence microscopy · Nanoscopy · Förster resonance energy transfer (FRET) · Fluorescence lifetime imaging microscopy (FLIM)

S. Padilla-Parra (✉)
Department of Pediatrics, Infectious Diseases, Emory University,
2015 Uppergate Dr,
Atlanta, GA 30322, USA
e-mail: sergi.padilla@emory.edu

N. Auduge · M. Coppey-Moisan
Institut Jacques Monod, UMR 7592, CNRS,
Université Paris-Diderot,
75013 Paris, France

M. Tramier
CNRS, UMR 6061,
Institut Génétique et Développement de Rennes,
35043 Rennes, France

M. Tramier
Faculté de Médecine, Université Rennes 1, UEB, IFR 140,
35043 Rennes, France

M. Tramier (✉)
Institut de Génétique et Développement de Rennes,
UMR 6061 CNRS, Université Rennes 1,
Faculté de Médecine 2 avenue du Professeur
Léon Bernard CS 34317,
35043 Rennes Cedex, France
e-mail: marc.tramier@univ-rennes1.fr

Introduction

In recent decades, the use of fluorescence microscopy has become a very useful approach to ascertain protein–protein interactions both in vitro and in vivo. During this time, what can be considered as classic techniques in spectroscopy, such as fluorescence resonance energy transfer (FRET) (Förster 1948), have been extensively used in fluorescence microscopy to investigate many biological systems and particularly protein interactions (Periasamy and Day 1999; Haj et al. 2002; Nikolaev et al. 2010)

When studying protein–protein interactions by FRET, the fraction of interacting protein is the more relevant biological parameter to be followed. In contrast to standard based-intensity methods, fluorescence lifetime imaging microscopy (FLIM) applied to live cells to quantify FRET is the appropriate method because it allows determining independently the FRET efficiency (E) and the fraction of interacting donor (f_D). f_D quantification is particularly important, since it determines the percentage of the

interacting protein population, and makes it possible to follow the spatio-temporal evolution of the interaction under investigation. Usually, analyses based on the least-squares method to fit fluorescence data coming from single photon detection systems are employed to recover fluorescence kinetics parameters (i.e. least squares or the maximum entropy method). In FRET–FLIM experiments, f_D is related to the fluorescence decay behavior of the donor and its quantification is most of the time carried out by fit procedures based on non-linear least squares methods. This approach becomes difficult (1) when donors' decays are better fitted with a multi-exponential function, for example employing the cyan fluorescent protein (CFP) (Tramier et al. 2006); and (2) when the number of acquired photons is too low; here, a compromise between the acquisition times and the high accuracy of quantitative analysis is required and fitting becomes a difficult task.

In this review, we stress the importance of novel analyses based on “non-fitting” approaches which allow recovering of direct quantitative data employing fast acquisition times (at the second time scale, even using multi-lifetime donors) like the minimal fraction of interacting donor method (mf_D) (Padilla-Parra et al. 2008; Yamada et al. 2009) or the phasor plot approach (Digman et al. 2008; Caiolfa et al. 2007; Leray et al. 2009, 2011; Grecco et al. 2009, 2010). A high number of events (at least thousands of photons per pixel) are needed to get statistically reasonable values to recover the mechanistic parameters of the system (i.e. lifetimes, pre-exponential factors, FRET efficiency) applying fitting procedures; hence, the time required for such measurements is too long: in the order of minutes. Under these circumstances, the FRET signal is averaged out (in a biological system, the kinetics of the interaction should not be faster than the

acquisition time; within minutes, this situation is quite likely to occur). Observe that this “FLIM resolution” is not related to the optical resolution but to the impact of long acquisition times in the final lifetime image. On the contrary, since non-fitting approaches allow quantitative analysis of signals with considerably fewer events compared to the referred fitting techniques (Lakowicz 2006), they are more convenient when working with living cells. The minimal fraction of interaction approach and the phasor plot approach permit one to obtain a quantitative image with small amounts of photons per pixel (i.e. 100) by hypothesizing a two-population system (FRET and no-FRET); a situation that in biology is quite likely to occur.

Instrumentation

Different instrumentation can be utilized in order to acquire FLIM data. We focus here on time domain (TD) approaches, (other techniques based on frequency domain (FD) are not discussed here; the reader is directed, for example, to Kremers et al. 2008; Buranachai et al. 2008). For TD–FLIM, one can distinguish between two different detection systems: (1) time correlated single photon counting (TCSPC-) and time- and space-correlated single photon counting (TSCSPC)-based devices, and (2) sequential acquisition (such as TriM–FLIM) using a time-gated-based method. Figure 1 shows a simplified schematic of both approaches.

In Fig. 1a, one can see a TCSPC detection-based system (Becker et al. 1999; Tramier et al. 2002; Becker and Bergmann 2004; Angelier et al. 2005; Yasuda et al. 2006; Padilla-Parra et al. 2008). The fluorescence coming from the sample (excited with a pulsed laser tuned, for example,

Fig. 1 Schematic describing the principle of two FLIM methodologies: TCSPC and Sequential FLIM. **a** A pulsed laser is directed to the sample and the fluorescence emission collected counting every single photon that arrives with a particular delay; a histogram is then

obtained which corresponds to the fluorescence decay of the corresponding fluorophore. **b** In sequential acquisition systems, photons are collected directly from a particular region of the decay with the help of an intensified charged couple device camera

at 80 Mhz) is imaged with a high numerical aperture objective and is directed to a single photon-counting detector. The detector converts the incident photon, coming from the fluorescence emission light of the sample. For time correlation, a time-to-amplitude converter is normally used that combines the information on the delay between the signal coming from the detector and the pulsed excitation recovered with a fast photodiode. A histogram describing the fluorescence decay for each pixel of an image is then obtained, giving rise to the FLIM image.

The sequential acquisition approach (Fig. 1b) is based on the detection system using a gated light intensifier coupled to a charged-couple device (CCD) camera (Owen et al. 2007; Padilla-Parra et al. 2008; Grant et al. 2008). The gate of the intensifier (adjusted to 1 or 2 ns depending on the experiment) is triggered by an electronic signal coming from the pulsed laser and a programmable delay box, together used to acquire a stack of time-correlated images, in which each image corresponds to a particular time delay of the fluorescence decay.

FLIM data analysis: non-fitting-based methods

Many different methods are at hand in order to analyze fluorescence intensity decay profiles: the method-of-moments, Laplace transformation, the maximum entropy method, Prony's method, sine transform, phase-plane approaches and the non-linear least squares method (NL-LSM). Above all, the last one is by far the most used nowadays because it helps to resolve complex decays for biological applications, even when the number of photons detected is relatively small. Normally NL-LSM is applied to TCSPC data assuming different mathematical models. The complete evaluation of the least squares analysis of fluorescence data has been discussed in depth in the literature (Lakowicz 2006). Finally, a promising method based on Bayesian analysis (Rowley et al. 2011) has recently been developed. By taking relatively low numbers of photons (50 counts per pixel), the authors showed that this approach is quite accurate for mono-exponential fluorophores (comparable to maximum likelihood, least squares, and phasor analysis). Further developments are, however, still needed in the context of multi-exponential situations to test its applicability for protein–protein interactions.

In this section, we review novel non-fitting-based methods: the minimal fraction of interacting donor (mf_D) approach (Padilla-Parra et al. 2008) and the phasor approach (Digman et al. 2008). It must be noted that a third technique is not extensively considered: the rapid lifetime determination (Woods et al. 1984; Ballew and Demas 1989; Sharman et al. 1999). RLD is another non-

fitting technique widely used for fast-FLIM applications. RLD has been applied in the context of fast-FLIM, especially for fluorophores exhibiting mono-exponential decays by taking only two time-windows (Elson et al. 2004; Requejo-Isidro et al. 2004); however, its applicability for multi-exponential decays and hence quantitative FLIM is still challenging and therefore out of the scope of this review (which is more focused on quantitative methods for FRET–FLIM).

The mean lifetime approach applied to Fast-FLIM acquisitions

The mean lifetime calculation is an alternative approach to quantitatively analyze a given fluorescence decay and is defined as

$$\langle \tau \rangle = \int t \times i(t) dt / \int i(t) dt \quad (1)$$

In which t is the time and $i(t)$ the intensity of the corresponding fluorescence signal. Considering a discrete sampling, the last expression can be resolved numerically resulting in

$$\langle \tau \rangle = \sum \Delta t_i \cdot I_i / \sum I_i \quad (2)$$

in which Δt_i is the time position of the corresponding time-gated window and I_i the intensity. Observe that a given stack of time-gated images on which the last expression is applied pixel by pixel could be obtained either by acquiring on-line with a fast FLIM system (i.e. fast-gated CCD camera (Hartmann and Ziegler 1996) or confocal time-gated detection (Sytsma et al. 1998)) or treating off-line a decay acquired with a system equipped with a TCSPC detector (Chang and Mycek 2010). Considering the accuracy and deviation of lifetime measurement, if we consider an infinite number of counted photons, the best measurement will be done using time channels of as few ps as with TCSPC devices. But the signal to noise ratio and the size of the time channels has to be considered simultaneously. By increasing the time window, the number of counted photons in each channel is increased and the deviation of the measurement is diminished. For live cell measurements, we have adjusted the measurement to five time-gated images of 2 ns channel width; in this situation, the deviation is reasonable and the accuracy is sufficient.

For FRET quantification, if one hypothesizes a biological two-population system (FRET and no-FRET) for a multi-lifetime donor, as is generally the case with a few exceptions (such as EGFP or mTFP1; Peter et al. 2005; Padilla-Parra et al. 2008, 2009; Ai et al. 2006; Walther et al. 2011), with a narrow distribution of

possible FRET efficiencies (E) one can write the next expression:

$$i(t) = f_D \sum a_i \times \exp(-t/\tau_{Fi}) + (1 - f_D) \times \sum a_i \exp(-t/\tau_{Di}) \quad (3)$$

In which f_D is the fraction of interacting donor, a_i the pre-exponential factors of different τ_{Di} lifetime species of the donor, and τ_{Fi} the corresponded FRET lifetime.

By using Eq. 3 in Eq. 1, if we isolate f_D , we have

$$f_D = [1 - (\langle \tau \rangle / \langle \tau_{>D} \rangle)] / \{1 - (\langle \tau \rangle / \langle \tau_{>D} \rangle) + [((\langle \tau \rangle / \langle \tau_{>D} \rangle) - (\langle \tau_{>F} \rangle / \langle \tau_{>D} \rangle)) / K]\} \quad (4)$$

where $\langle \tau_{>D} \rangle = \sum a_i \cdot \tau_{Di}^2 / \sum a_i \cdot \tau_{Di}$, $\langle \tau_{>F} \rangle = \sum a_i \cdot \tau_{Fi}^2 / \sum a_i \cdot \tau_{Fi}$ and $K = \sum a_i \cdot \tau_{Di} / \sum a_i \cdot \tau_{Fi}$. Eq. 4 is not really useful since all the discrete lifetimes τ_{Di} and τ_{Fi} have to be known. But, we introduced the minimization of f_D (mf_D). f_D depends on three variables ($\langle \tau_{>F} \rangle / \langle \tau_{>D} \rangle$, $\langle \tau \rangle / \langle \tau_{>D} \rangle$ and K). In order to obtain mf_D , we minimize f_D following K and $\langle \tau_{>F} \rangle / \langle \tau_{>D} \rangle$ (for details, see Padilla-Parra et al. 2008). One obtains a general equation that can be applied to multi-lifetime donors and is particularly useful in biology:

$$mf_D = 1 - \langle \tau \rangle / \langle \tau_{>D} \rangle \quad (5)$$

In the particular case of a chromophore which plays the role of the donor, it can be fitted to a single exponential model and Eq. 3 becomes

$$i(t) = f_D \times \exp(-t/\tau_F) + (1 - f_D) \times \exp(-t/\tau_D) \quad (6)$$

Then, using Eq. 6 in Eq. 1 and isolating f_D , we get

$$f_D = [1 - (\langle \tau \rangle / \tau_D)] / [1 - (\langle \tau \rangle / \tau_D) - (\tau_F / \tau_D)^2 + (\langle \tau \rangle / \tau_D) \times (\tau_F / \tau_D)] \quad (7)$$

In a similar approach, minimization of f_D following τ_F / τ_D (for details, see Padilla-Parra et al. 2008) results in a minimal fraction of interacting donor expression:

$$mf_D = [1 - (\langle \tau \rangle / \tau_D)] / [(\langle \tau \rangle / 2 \times \tau_D) - 1]^2 \quad (8)$$

Observe that in Eqs. 5 and 8 τ_D (or $\langle \tau_{>D} \rangle$) is the lifetime of donor alone. This means that, regardless of the behavior of the donor (adjustable either to a multiple exponential model, Eq. 4, or to a single exponential, Eq. 8) with a single experiment, and utilizing very fast acquisition times, we have access to the mean lifetime, an apparent FRET efficiency which depends on the mean lifetime of the isolated donor and the mean lifetime of donor in the presence of acceptor, and a minimal fraction of interacting donors which is related to the relative concentration (Padilla-Parra et al. 2010). Moreover, spatio-temporal information on all these parameters can be obtained with a very good time resolution. The mf_D approach only depends upon experimental parameters (i.e. $\langle \tau \rangle$ and τ_D) and can be applied straightforwardly pixel by pixel obtaining instantaneously the corresponding quantitative mf_D micrograph. As an example, one can observe in Fig. 2

Fig. 2 mf_D and mean lifetime calculation. In the left panels the intensity and mean lifetime FLIM images are presented for a cell expressing EGFP-H4 alone and a cell co-expressing BD-eGFP in the presence of H4-mCherry. In a three-dimensional representation the mf_D shows the localization and extent of the interaction (orange peaks)

the interaction between Histone H4 tagged to mCherry and the Bromodomain TAF_{II}250 tagged to EGFP.

It is interesting to highlight that mf_D can be applied to both TCSPC detection-based systems and sequential acquisition systems. mf_D can be recovered on-line during acquisition since the computation of $\langle\tau\rangle$ and mf_D is straightforward, regardless of the behavior of the donor (applying the corresponding model for single or multi-lifetime donor fluorophores). This is a major advantage when studying protein interactions in live cells, since the user has quantitative information immediately at hand.

The phasor plot approach

The phasor plot approach (Fig. 3) is also interesting in terms of fast acquisitions, since no fitting is required in order to obtain quantitative results. One of the most attractive features of phasor plot analysis is that, with this type of 2D representation, one can take into consideration the contribution of the background and/or auto-fluorescence.

The phasor plot representation is based on a 2D histogram in which each pixel of an image has a vector assigned to it (Fig. 3a), defined by the sine-cosine

Fig. 3 Phasor plot. **a** A schematic consisting in two coordinates defined in Eqs. 1, 2 shows typical regions for single exponential donor (blue circle), Hypothetical 100% FRET efficiency (green circle) and autofluorescence (red circle). **b** Phasor plot corresponding to a solution of Rhodamine 6 G; all phasors fall in the universal circle as expected. Phasor plot to quantify protein interactions. **c** The phasor plot approach is here utilized to detect the interaction between Histone H4 tagged with mCherry and the bromodomain (BD) of the acetyl-transferase TAF₂₅₀ tagged with eGFP in HEK293T cells. This interaction is seen because the red circle is shifted toward the right-hand side (4th panel) compared to the control BD-eGFP alone (2nd panel). The 1st and 3rd panels show the pixels which correspond to the selected phasors within the red circle

transform, (Digman et al. 2008) with two coordinates (s and g):

$$g_{i,j}(w) = m_{i,j} \cos(\varphi_{i,j}) \quad (9)$$

$$s_{i,j}(w) = m_{i,j} \sin(\varphi_{i,j}) \quad (10)$$

In these equations, $m_{i,j}$ and $\varphi_{i,j}$ are the modulation and phase relative to the excitation for each pixel of the related image

This approach can also be applied to time domain data for which the next equations apply:

$$g_{i,j}(w) = \int I_{i,j}(t) \cos(wt) dt / \int I_{i,j}(t) dt \quad (11)$$

$$s_{i,j}(w) = \int I_{i,j}(t) (\sin)(wt) dt / \int I_{i,j}(t) dt \quad (12)$$

$I_{i,j}(t)$ is the fluorescent intensity decay for every pixel of a given image and ω is the laser frequency used. Fig. 3a shows a schematic example of the polar plot. The blue circle represents all points of a given image for a fluorophore playing the role of the donor in a FRET experiment. One can also see that the blue circle lies on the black semicircle that represents, according to the expressions for the calculation of the phasor in a single-exponential situation (Digman et al. 2008), the “universal circle” of all possible single lifetimes ($\tau_D = \tau_\varphi = \tau_M$) for an interacting fluorophore. When FRET occurs, the corresponding phasor will shift towards the right-hand side of the plot, and in this case, the green circle represents an ideal FRET situation in which the fractional contribution of the quenched signal is 100%, and therefore the short lifetime prevails ($\tau_F = \tau'_\varphi = \tau'_M$). The blue line between the blue and green circles represents the fractional contribution of each lifetime. In Fig. 3b, an example usually employed to calibrate the system is shown: Rhodamine 6 G. The blue circle is placed on the universal circle, and this underscores the single exponential behavior of this fluorophore.

The strength points of this method are (1) one can compare a big set of different analyses (i.e. different cells in different conditions), and (2) it allows the discrimination of the FRET signal from the auto-fluorescence (both phenomena fall in different regions of the phasor plot), and finally (3) the phasor plot allows data from different devices [both in time domain (TCSPC-based systems and sequential acquisition with a CCD camera) and frequency domain systems]. In Fig. 3b is a biological example based on histone acetylation (Padilla-Parra et al. 2008). The acquisition was carried out taking 21 time-gated images of 1-ns delay each. HEK293 cells expressing mCherry-H4 alone

(left panel, control) and in the presence of BD-EGFP (right panel, FRET) were employed for this example. It can also be said that, in this situation, a significant reduction in the lifetime was previously calculated, and the phasor approach was able to reproduce the same qualitative results (detection of the interaction and localization) as the minimal fraction of interacting donor (Fig. 2). Observe that both analyses are equivalent in terms of localization of the FRET signal (top right panel Fig. 3b highlighted in red, compared to the co-transfected FLIM image and also the mf_D image in Fig. 2). All points coming from uncorrelated pixels will fall in the lower region of the plot, allowing the user to distinguish between the contributions of FRET and auto-fluorescence. However, the quantitative results with the phasor approach were not found since multiple solutions were found for the position of the same spot in terms of FRET efficiency, percentage of background and percentage of donor unquenched ($1 - f_D$). The fact that one finds multiple solutions for one spot represents a caveat for this approach.

Outlook

The development of non-fitting models is crucial to studying the spatio-temporal dynamics of a given protein–protein interaction. Putting aside the loss of spatial resolution of any FLIM micrograph obtained acquiring ~2 or 3 min, the study of the temporal dynamics of protein interactions requires speeding up the process of data collection. This increase in acquisition speed leads to a limited number of photons per pixel, and therefore fitting-based approaches fail to produce reliable data in situations in which only a few photons per pixel are available (i.e. 100). Thanks to the development of non-fitting approaches like the phasor plot and the minimal fraction of interacting donor approach (for single and multi-lifetime donors) easily applicable to both systems: (1) sequential acquisition using time-gated images (such as Trim-FLIM; LaVision Biotech, Bielefeld, Germany) and (2) TCSPC detector-based systems [both wide-field (Europhoton, Berlin, Germany) and confocal scanning microscopy (Becker and Hickl, Berlin, Germany and Picoquant, Berlin, Germany)] it is possible to create movies in which a certain protein interaction can be quantitatively followed as a function of time (Padilla-Parra et al. 2010). The possibility to obtain FLIM movies in which the FRET phenomenon is quantified gives rise to a field of study in which image correlation techniques could directly be applied on quantitative data. Several authors have already presented results in the form of a time-lapse in FLIM studies (Clayton et al. 2002; Cremazy et al. 2005; Gadella and Jovin 1995; Jares-Erijman and Jovin 2003; Pepperkok et al. 1999; Lee et al. 2009). Moreover, Rickman and Duncan (2010) followed the dynamics of

syntaxin and Munc18 at the plasma membrane targeting the laser excitation beam at the membrane and acquiring the evolution of the fluorescence decay with a total acquisition time of 100 ms; it must be said that in this experiment the information was restricted to one single point. Nevertheless, few studies have been conducted in which the time resolution between frames is short enough to facilitate following the interaction spatio-temporally in the whole image focusing on the quantification of the interaction and not only in lifetime analysis.

Other approaches to quantify FRET by FLIM based on global analysis like the acceptor rise (Laptenok et al. 2010), or the phasor plot combined with global analysis improving the signal-to-noise ratio (Grecco et al. 2009) are also very promising. In that respect, Laptenok and co-workers quantified FRET by using the rise time of the acceptor, hence they increased the precision of their measurements since their approach globally correlates donor lifetime diminution with acceptor lifetime rise; however, total acquisition times in order to obtain reliable data to fit all curves globally were longer than 2 min (150 s).

Altogether, this mini-review highlights the importance of non-fitting approaches for quantitative FRET–FLIM in the context of quantifying protein–protein interactions in single living cells. Interestingly, these non-fitting approaches are compatible with very fast acquisitions since high photon statistics are not required in order to obtain a close estimate of the fraction of interacting donor (i.e. mf_D).

Acknowledgement This work was supported by the Fondation pour la Recherche Médicale, the Région Ile de France (Soutien aux Equipes Scientifiques pour l'Acquisition de Moyens Expérimentaux), the Association Nationale pour le Recherche (ANR-PFTV) (to M, C.-M.), IBISA (to M, C.-M, and M.T.), Rennes Métropole and Région Bretagne (to M.T.).

References

- Ai HW, Henderson JN, Remington SJ, Campbell RE (2006) Directed evolution of a monomeric, bright and photostable version of *Clavularia* cyan fluorescent protein: structural characterization and applications in fluorescence imaging. *Biochem J* 400:531–540
- Angelier N, Tramier M, Louvet E, Coppey-Moisand M, Savino TM, De Mey JR, Hernandez-Verdun D (2005) Tracking the interactions of rRNA processing proteins during nucleolar assembly in living cells. *Mol Biol Cell* 16:2862–2871
- Ballew R, Demas JN (1989) An error analysis of the rapid lifetime determination method for the evaluation of single exponential decays. *Anal Chem* 61:30–33
- Becker W, Bergmann A (2004) Multi-dimensional time-correlated single photon counting. In: Gedes CD, Lackowicz JR (eds) *Review in fluorescence* 2005. Springer, New York
- Becker W, Hickl H, Zander C, Drexhage KH, Sauer M, Siebert S, Wolfrum J (1999) Time resolved detection and identification of single analyte molecules in microcapillaries by time-correlated single photon counting (TCSPC). *Rev Sci Instrum* 70:1835–1841
- Buranachai C, Kamiyama D, Chiba A, Williams BD, Clegg RM (2008) Rapid frequency-domain FLIM spinning disk confocal microscope: lifetime resolution, image improvement and wavelet analysis. *J Fluoresc* 18:929–942
- Caiola VR, Zamai M, Malengo G, Andolfo A, Madsen CD, Sutin J, Digman MA, Gratton E, Blasi F, Sidenius N (2007) Monomer dimer dynamics and distribution of GPI-anchored uPAR are determined by cell surface protein assemblies. *J Cell Biol* 179(5):1067–1082
- Chang CW, Mycek MA (2010) Precise fluorophore lifetime mapping in live-cell, multi-photon excitation microscopy. *Opt Express* 18:8688–8696
- Clayton AHA, Hanley QS, mdt-Jovin DJ, Subramaniam V, Jovin TM (2002) Dynamic fluorescence anisotropy imaging microscopy in the frequency domain (rFLIM). *Biophys J* 83:1631–1649
- Cremazy FGE, Manders EMM, Bastiaens PIH, Kramer G, Hager GL, van Munster EB, Verschure PJ, Gadella TWJ, van Driel R (2005) Imaging in situ protein-DNA interactions in the cell nucleus using FRET-FLIM. *Exp Cell Res* 309:390–396
- Digman MA, Caiola VR, Zamai M, Gratton E (2008) The phasor approach of fluorescence lifetime imaging analysis. *Biophys J* 94:L14–L16 b
- Elson D, Requejo-Isidro J, Munro I, Reavell F, Siegel J, Suhling K, Tadrous P, Benninger R, Lanigan P, McGinty J, Talbot C, Treanor B, Webb S, Sandison A, Wallace A, Davis D, Lever J, Neil M, Phillips D, Stamp G, French P (2004) Time-domain fluorescence lifetime imaging applied to biological tissue. *Photochem Photobiol Sci* 3:795–801
- Förster T (1948) Intermolecular energy migration and fluorescence. *Ann Phys* 6:55–75
- Gadella TWJ, Jovin TM (1995) Oligomerization of epidermal growth-factor receptors on A431 cells studied by time-resolved fluorescence imaging microscopy - a stereochemical model for tyrosine kinase receptor activation. *J Cell Biol* 129:1543–1558
- Grant DM, Zhang W, McGhee EJ, Bunney TD, Talbot CB, Kumar S, Munro I, Dunsby C, Neil MA, Katan M, French PM (2008) Multiplexed FRET to image multiple signaling events in live cells. *Biophys J* 95:L69–L71
- Grecco HE, Roda-Navarro P, Verveer PJ (2009) Global analysis of time correlated single photon counting FRET-FLIM data. *Opt Express* 17:6493–6508
- Grecco HE, Roda-Navarro P, Girod A, Hou J, Frahm T, Truxius DC, Pepperkok R, Squire A, Bastiaens PI (2010) In situ analysis of tyrosine phosphorylation networks by FLIM on cell arrays. *Nat Methods* 7:467–472
- Haj FG, Verveer PJ, Squire A, Neel BG, Bastiaens PIH (2002) Imaging sites of receptor dephosphorylation by PTP1B on the surface of the endoplasmic reticulum. *Science* 1708–1711
- Hartmann P, Ziegler W (1996) Lifetime imaging of luminiscent oxygen sensors based on all-solid-state technology. *Anal Chem* 68:4512–4514
- Jares-Erijman EA, Jovin TM (2006) Imaging molecular interactions in living cells by FRET microscopy. *Curr Opin Chem Biol* 10:409–416
- Kremers GJ, van Munster EB, Goedhart J, Gadella TW Jr (2008) Quantitative lifetime unmixing of multiexponentially decaying fluorophores using single-frequency fluorescence lifetime imaging microscopy. *Biophys J* 95:378–389
- Lakowicz JR (2006) *Principles of fluorescence spectroscopy*, 3rd edn. Springer, New York
- Laptenok SP, Borst JW, Mullen KM, van Stokkum IH, Visser AJ, van Amerongen H (2010) Global analysis of Förster resonance energy transfer in live cells measured by fluorescence lifetime imaging microscopy exploiting the rise time of acceptor fluorescence. *Phys Chem Chem Phys* 12:7593–75602
- Lee SJR, Escobedo-Lozoya Y, Szatmari EM, Yasuda R (2009) Activation of CaMKII in single dendritic spines during long-term potentiation. *Nature* 458:299–304

- Leray A, Spriet C, Trinel D, Héliot L (2009) Three-dimensional polar representation for multispectral fluorescence lifetime imaging microscopy. *Cytom A* 75:10749–11014
- Leray A, Spriet C, Trinel D, Blossy R, Usson Y, Héliot L (2011) Quantitative comparison of polar approach versus fitting method in time domain FLIM image analysis. *Cytom A* 79:149–158
- Nikolaev VO, Moshkov A, Lyon AR, Miragoli M, Novak P, Paur H, Lohse MJ, Korchev YE, Harding SE, Gorelik J (2010) β_2 -adrenergic receptor redistribution in heart failure changes cAMP compartmentation. *Science* 1653–1657
- Owen DM, Auksoorius E, Manning HB, Talbot CB, de Beule PA, Dunsby C, Neil MA, French PM (2007) Excitation-resolved hyperspectral fluorescence lifetime imaging using a UV-extended supercontinuum source. *Opt Lett* 32:3408–3410
- Padilla-Parra S, Audugé N, Coppey-Moisson M, Tramier M (2008) Quantitative comparison of different fluorescent protein couples for fast FRET-FLIM acquisition. *Biophys J* 97:2368–2376
- Padilla-Parra S, Audugé N, Lalucque H, Mevel JC, Coppey-Moisson M, Tramier M (2009) Quantitative comparison of different fluorescent protein couples for fast FRET-FLIM acquisition. *Biophys J* 97:2368–2376
- Padilla-Parra S, Audugé N, Tramier M, Coppey-Moisson M (2010) Hetero-FRET imaging by time domain FLIM: a quantitative method to follow transient protein-protein interactions in living cells. Cold Spring Harbor Press, New York
- Pepperkok R, Squire A, Geley S, Bastiaens PIH (1999) Simultaneous detection of multiple green fluorescent proteins in live cells by fluorescence lifetime imaging microscopy. *Curr Biol* 9:269–272
- Periasamy A, Day RN (1999) Visualizing protein interactions in living cells using digitized GFP imaging and FRET microscopy. *Methods Cell Biol* 58:293–314
- Peter M, Ameer-Beg SM, Hughes MKY, Keppler MD, Prag S, Marsh M, Vojnovic B, Ng T (2005) Multiphoton-FLIM quantification of the EGFP-mRFP1 FRET pair for localization of membrane receptor-kinase interactions. *Biophys J* 88:1224–1237
- Requejo-Isidro J, McGinty J, Munro I, Elson DS, Galletly NP, Lever MJ, Neil MAA, Stamp GWH, French PMW, Kellett PA, Hares JD, Dymoke-Bradshaw AKL (2004) High-speed wide-field time-gated endoscopic fluorescence-lifetime imaging. *Opt Lett* 29:2249–2251
- Rickman C, Duncan RR (2010) Munc18/Syntaxin interaction kinetics control secretory vesicle dynamics. *J Biol Chem* 285:3965–3972
- Rowley M, Barber PR, Coolen ACC, Vojnovic B (2011) Bayesian analysis of fluorescence lifetime imaging data. *Proc SPIE* 7903:790325
- Sharman KK, Periasamy A, Ashworth H, Demas JN, Snow NH (1999) Error analysis of the rapid lifetime determination method for double-exponential decays and new windowing schemes. *Anal Chem* 71:947–952
- Sytsma J, Vroom JM, De Grauw CJ, Gerritsen HC (1998) Time-gated fluorescence lifetime imaging scanning microscopes: acquisition speed, photon economy and lifetime resolution. *J Microsc* 206:218–224
- Tramier M, Gautier I, Piolot T, Ravalet S, Kemnitz K, Coppey J, Durieux C, Mignotte V, Coppey-Moisson M (2002) Picosecond-hetero-FRET microscopy to probe protein-protein interactions in live cells. *Biophys J* 83:3570–3577
- Tramier M, Zahid M, Mevel JC, Masse MJ, Coppey-Moisson M (2006) Sensitivity of CFP/YFP and GFP/mCherry pairs to donor photobleaching on FRET determination by fluorescence lifetime imaging microscopy in living cells. *Microsc Res Tech* 11:933–942
- Walther KA, Papke B, Sinn MB, Michel K, Kinkhabwala A (2011) Precise measurement of protein interacting fractions with fluorescence lifetime imaging microscopy. *Mol Biosyst* 7:322–336
- Woods RJ, Scypinski S, Cline Love LJ (1984) Transient digitizer for the determination of microsecond luminescence lifetimes. *Anal Chem* 56:1395–1400
- Yamada H, Padilla-Parra S, Park SJ, Itoh T, Chaineau M, Monaldi I, Cremona O, Benfenati F, De Camilli P, Coppey-Moisson M, Tramier M, Galli T, Takei K (2009) Dynamic interaction of amphiphysin with N-WASP regulates actin assembly. *J Biol Chem* 284:34244–34256
- Yasuda R, Harvey CD, Zhong HN, Sobczyk A, van Aelst L, Svoboda K (2006) Supersensitive Ras activation in dendrites and spines revealed by two-photon fluorescence lifetime imaging. *Nat Neurosci* 9:283–291