Available online at www.sciencedirect.com
ScienceDirect
Current Opinion in Biotechnology

Fluorescence lifetime and polarization-resolved imaging in cell biology

James A Levitt[1], Daniel R Matthews[2], Simon M Ameer-Beg[2,3] and Klaus Suhling[1]

Fluorescence lifetime imaging (FLIM) and fluorescence polarization imaging are complementary techniques that can be used to extract information about macromolecules from biological samples. Owing to the sensitivity of fluorescence to the physicochemical environment, and nanometer-scale interactions via Förster resonance energy transfer (FRET), FLIM has been implemented in many laboratories for numerous applications in the life sciences and beyond. This review seeks to provide a brief overview of some of the recent advances in the techniques and more pertinently their applications in cell and tissue imaging. The particular merits of polarization-resolved fluorescence measurements are highlighted, including the unique ability to elucidate the occurrence of homo-FRET.

Addresses
[1] Department of Physics, King's College London, Strand, London WC2R 2LS, UK
[2] Randall Division for Cell and Molecular Biophysics, King's College London, New Hunt's House, Guys Campus, London SE1 1UL, UK
[3] Dimbleby Department of Cancer Research, Division of Cancer Studies, King's College London, New Hunt's House, Guys Campus, London SE1 1UL, UK

Corresponding author: Levitt, James A (james.levitt@kcl.ac.uk), Matthews, Daniel R (daniel.matthews@kcl.ac.uk), Ameer-Beg, Simon M (simon.ameer-beg@kcl.ac.uk) and Suhling, Klaus (klaus.suhling@kcl.ac.uk)

Current Opinion in Biotechnology 2009, **20**:28–36

This review comes from a themed issue on
Analytical biotechnology
Edited by Christopher Contag and Kevin Eliceiri

Available online 4th March 2009

0958-1669/$ – see front matter

DOI 10.1016/j.copbio.2009.01.004

Introduction

Optical imaging techniques, in particular fluorescence imaging techniques, are powerful tools in the biological and biomedical sciences, because they are minimally invasive and can be applied to living cells and tissues. Fluorescence can be characterized by multiple parameters, including intensity, wavelength, lifetime and polarization. For its application to biological studies involving cells, fluorescence is often measured in an imaging modality, where the contrast in the image can be generated by these parameters that is, by fluorescence intensity, wavelength, lifetime or polarization, or combinations thereof. The fluorescence detection sensitivity extends down to the single molecule level, and sophisticated chemistry or bioengineering provides an ever-growing palette of colours afforded by fluorescent probes [1,2].

FLIM, in which contrast in the image is provided by spatial variations in fluorescence lifetime of the probe has become established as an extremely powerful technique in the physical and life sciences [3,4••]. The image contrast is largely concentration and intensity independent but is sensitive to the environmental surroundings of the fluorophore, for example pH [5,6], refractive index [7,8] or viscosity [9]. In the absence of labels, contrast can also be afforded using the inherent autofluorescence of many samples, which is of interest for clinical diagnostics, for example cancer detection and treatment [10,11]. In cell biology FLIM is commonly used for measuring protein–protein interactions via Förster resonance energy transfer (FRET) using molecules labelled with appropriate dyes or fluorescent proteins [12]. The widespread use of the FRET-FLIM technique means that careful calibration and data evaluation in FRET experiments are necessary for reliable FRET quantification [13–15].

Instrumentation

Typically, FLIM measurements are made using either single photon or multiphoton excitation from laser sources, often Ti:Sapphire lasers, or low-cost light-emitting diodes (LEDs). The recent availability of compact broadband supercontinuum sources allows excitation across the wavelength range of the visible region with a reduced complexity of the experimental arrangement, and FLIM can be performed using LEDs for both time [16] and frequency domain [17] measurements. The signal can be detected with a photomultiplier or diode in scanning systems, and with a gated or modulated intensified camera in wide-field imaging systems. While wide-field systems are fast, time-correlated single photon counting (TCSPC)-based FLIM with scanning systems has an excellent signal-to-noise ratio [18•], provides intrinsic optical sectioning and is easily combined with spectral detection. Recent technical advances have allowed for more rapid acquisition in multiphoton FLIM by the use of multiple scanning beams [13,19,20] and spinning (Nipkow) discs [21,22]. The reduction in acquisition times may provide the

opportunity to study dynamic processes in cells and is an important step towards high content/throughput screening for specific protein–protein interactions for drug discovery [23].

Spectrally resolved FLIM

Spectrally resolved fluorescence lifetime imaging (SLIM [24] or SFLIM [25]) and multispectral FLIM [26,27] record the lifetime and spectrum of the fluorescence in each pixel of an image and have been used to study autofluorescence from unstained tissue. Such multidimensional imaging with parallel acquisition allows simultaneously for additional contrast in the recorded image and identification of multiple species. Most of these systems work with beam scanning, but a streak camera-based system for simultaneous acquisition of fluorescence decay and spectrum in 9 or 16 pixels using a microlens array and stage-scanning for multiphoton excitation has also been demonstrated [28,29]. This system has been used for imaging dyes in cells and autofluorescence imaging of cells.

Applications

The FRET-FLIM technique is becoming increasingly popular for the study of protein–protein interactions in living cells, as it allows for the detection of the proximity of two fluorescent probes well below the diffraction-limited optical resolution [12]. For example, FLIM has recently been used to study the different interactions of green fluorescent protein (GFP)-labelled fibroblast growth factor receptors (FGFR) FRS2 (FGFR substrate 2) with a monomeric red fluorescent protein (mRFP)-labelled wild type and a mutant receptor [30]. Different interactions were observed after a single point mutation, thus introducing a higher level of complexity into the efforts to understand cell signalling and complicating systems biology interpretations. FLIM has also recently been used to elucidate the presence of microclusters of phosphorylated inhibitory killer immunoglobulin-like receptors (KIR) in immunological synapses. Using a GFP-tagged receptor and Cy3-tagged antibody, FRET was observed at the synapse [34]. The technique has been used to study integrin-effector binding using α4 and β1 integrin in a quantitative manner using a GFP-mRFP FRET pair [35]. In the study FRET-FLIM was used to give information on how the conformational state of the receptor may determine the activity of anti-integrin molecules, by using assays capable of detecting integrin-effector binding. The technique may have potential for screening assays of small molecule inhibitors. A ratiometric chloride indicator Clomeleon based on cyan fluorescent protein (CFP) and Topaz, a variant of yellow fluorescent protein (YFP), was used for FRET-FLIM as part of a study of neuronal development by monitoring intracellular chloride concentrations. It was found that the FRET signal correlated well with the neuronal development, with the Clomeleon lifetime reporting on the concentration of chloride in neurons [36]. A different FRET pair, Cerulean and Citrine has been used to monitor synaptic interactions involving the presynaptic protein Bassoon in COS-7 cells and hippocampal neurons [37]. By using FRET-FLIM the importance of Bassoon for the formation of a macromolecular complex by corecruitment of two interaction partner proteins was determined. This FRET pair was also utilized for monitoring interactions between elongin B and elongin C, which form a complex with the van Hippel-Landau (VHL) gene product. By labelling elongin C with both Cerulean and Citrine at the N-terminus and C-terminus, respectively, a small increase in the intramolecular FRET efficiency was observed, suggestive of a conformational change in elongin C when co-expressed with elongin B [38]. Developments in acceptor chromophores, including acceptors that do not fluoresce [39], are allowing for more sophisticated assays to be implemented. These ''dark'' acceptors permit the use of a larger spectral window for measuring donor fluorescence by removing the complications associated with the overlap of donor and acceptor emission. Furthermore, the spectral window left available by the absence of the acceptor emission can be utilized for other probes in multiplexed experiments. Multiplexing for FRET experiments has also been demonstrated using the FLIM-FRET of a TagRFP/mPlum FRET pair alongside spectral ratiometric imaging of an ECFP/Venus pair. Using these two FRET pairs the authors were able to measure the calcium flux and GTP-ase activation simultaneously in cells [40•]. Other recent developments include super-resolution FLIM with ultra-high spatial resolution using stimulated emission depletion (STED) microscopy [31], for studies of biological processes on length scales below the classical diffraction limit, and also the combination of FLIM with microfluidics [32,33].

Polarization-resolved fluorescence imaging

Polarization measurements can yield information on the properties of a sample that cannot be extracted by simple intensity and lifetime methods, as summarized in Table 1. These include molecular orientation, energy migration (homo-FRET), and rotational diffusion that can all lead to a depolarization of the emitted fluorescence. For these measurements it is most useful to discuss polarization in terms of the fluorescence anisotropy, r, as defined by the Jablonski-Perrin equation for isotropic samples:

$$\frac{1}{r} = \frac{1}{r_0}(1 + 6D_{rot}\tau_f) \quad (1)$$

where r_0 is the initial anisotropy, a fixed molecular property given by the orientation of the absorption and emission transition dipole moments, D_{rot} is the rotational diffusion coefficient that can be used to deduce the hydrodynamic volume of a fluorescent molecule or the viscosity of the surrounding medium and τ_f is the fluorescence lifetime.

Table 1

Biologically relevant benefits/uses of polarization-resolved fluorescence measurements	References
Potentially higher dynamic range than lifetime measurements for hetero-FRET experiments in typical time-domain FLIM, provided a sufficient number of photons are collected.	[15,53]
Use of one type of label makes larger spectral window open for other probes and multi-dimensional fluorescence measurements, plus reduced labelling effort	
Measurements of rotational mobility of fluorophores:	
Used for monitoring	
changes in nuclear morphology	[43]
membrane fluidity	[44]
digestion of DNA by nucleases.	[45]
Measurements of energy migration (or homo-FRET):	
Determination of	
cluster sizes of GFP-labelled lipid markers	[59]
oligomerization states/degree of oligomerization	[60••]
heterogeneity of lipid order on nano-scale in plasma membrane.	[58,60••]

The molecular tumbling is described by the rotational correlation time, φ, for which use of the Stokes–Einstein equation gives

$$\varphi = (6D_{rot})^{-1} = \eta V/kT \quad (2)$$

where k is the Boltzmann constant, T is the absolute temperature, V is the molecular hydrodynamic volume of the fluorophore and η is the viscosity of the medium [41].

In practice the sample is excited using a linearly polarized light source, and the time-resolved intensities of the components of the fluorescence polarized parallel, $I_{//}(t)$, and perpendicular, $I_{\perp}(t)$, to the polarization vector of the excitation source are measured. The time-resolved fluorescence anisotropy is then

$$r(t) = \frac{I_{//}(t) - GI_{\perp}(t)}{I_{//}(t) + 2GI_{\perp}(t)} \quad (3)$$

where the factor G accounts for the differences in detection sensitivity between the two orthogonal polarizations, and the denominator is proportional to the total fluorescence intensity. The extent of depolarization of the fluorescence emission allows for the determination of the rotational motion, the nature of the environment and the occurrence of energy transfer. Owing to the nature of the photoselection process for absorption, multiphoton excitation can be used to provide a greater dynamic range for anisotropy measurements [42].

Anisotropy imaging

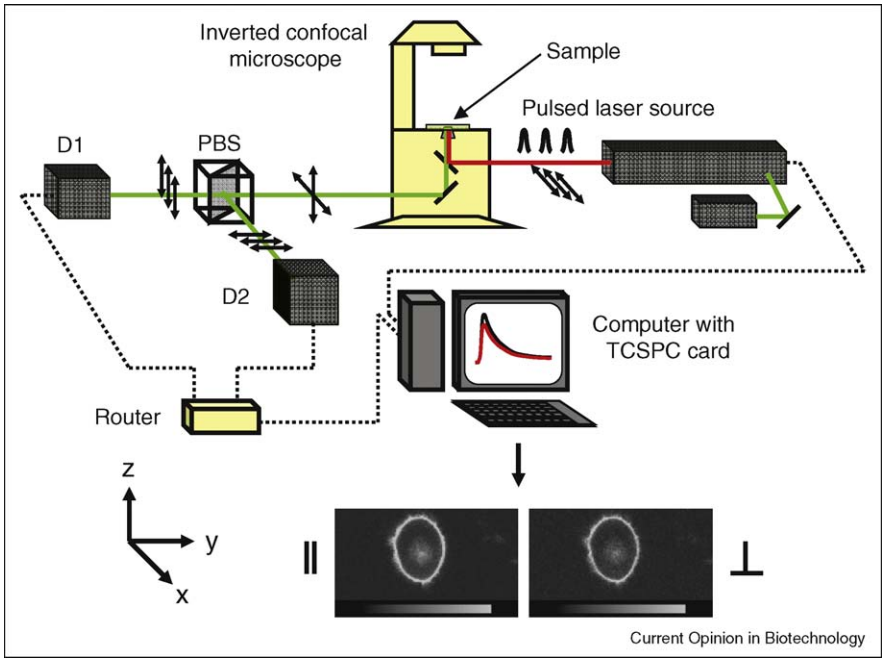
Anisotropy imaging can be performed as steady-state or time-resolved measurements in the time-domain or frequency-domain and using scanning or wide-field methods, as summarized in Table 2. The experimental geometry requires a polarizer, or polarizing beamsplitter, in the detection arm for single or two-detector (simultaneous) polarization-resolved measurements, respectively. The key advantage of using two detectors or a polarizing beamsplitter is the simultaneous acquisition of the orthogonal polarization components. This minimizes artefacts owing to photobleaching, movement or other changes in the sample as well as instrumental drift, and is also faster than two sequential acquisitions. For time-resolved measurements, the excitation source source must be modulated or pulsed, and the detection can be modulated, or based on time-gating or TCSPC. An example of the experimental arrangement for laser-scanning, time-resolved fluorescence anisotropy microscopy based on TCSPC is shown in Figure 1. In the case of microscopy, one must be aware of, and correct for, any depolarization that occurs in the optical train of the system, particularly when using high numerical aperture objectives, which can significantly reduce the r_0 value recovered.

Table 2

Fluorescence anisotropy imaging implementations.

Implementation/advantages	Steady state	Time-resolved
Scanning		
+ intrinsic optical sectioning	[63,64]	Time-domain TCSPC
+ easy spectral multiplexing		Frequency domain
Wide-field		
+ fast	[58]	Time-domain [47,48], [59••]
		Frequency domain [49]

Figure 1



Experimental arrangement for time-resolved fluorescence anisotropy imaging measurements using time-correlated single photon counting (TCSPC) detection. A linearly polarized pulsed laser source is used to excite the sample and the fluorescence is passed through a polarizing beamsplitter cube (PBS) with the orthogonal components of the polarization detected by two detectors (D1, D2) simultaneously. The outputs from the detectors are fed into a multi-channel router and into a TCSPC card. The parallel and perpendicular fluorescence decays are measured for each pixel. Arrows in the beam path indicate the polarization state of the light. Two polarization-resolved images are measured simultaneously and can be processed to generate an anisotropy image, or alternatively a region of interest may be selected and an anisotropy decay can be extracted using Eq. (3). From these data the rotational correlation time and viscosity, or molecular volume can be calculated using Eqs. (2) and (3).

Steady-state

Many steady-state studies have made use of the fact that small fluorescent molecules have an anisotropy value of close to zero in solution, because the right hand side of Eq. (1) is very large, due to the high value of the rotational diffusion coefficient. Any increases in measured r are typically due to a reduction in the rotational mobility of the fluorophore. Steady-state anisotropy imaging has been used to monitor the compaction states of chromatin with associated changes in the morphology of the nucleus of HeLa cells expressing H2B-GFP when treated with trichostatin-A (TSA) [43]. More densely packed chromatin results in a reduction in the rotational mobility of the labelled species and therefore higher anisotropy values. In another example, by monitoring the rotational dynamics via anisotropy imaging of FITC-CD44Ab labelled PG cells, the fluidity of the cell membrane upon stimulation of the cells using berberine has been measured [44]. TAMRA-labelled DNA injected into live cells from a breast cancer line MDA-MB-231 was measured using steady-state anisotropy imaging to monitor digestion of the DNA by nucleases in localized areas of the cell as a function of time [45]. A decrease in the anisotropy value from the nucleus and cytoplasm of live cells occurred as the labelled DNA fragments were cleaved. In this case, anisotropy measurements offer a distinct advantage over FRET assays. For the latter, there may be a sharp decrease in the FRET efficiency arising when one of the FRET pair becomes separated from the other during DNA digestion. However, the FRET pair may thus only be reporting on a single specific digestive cut whereas anisotropy measurements reflect a more extensive range of digestive cuts separating all labelled products into small oligonucleotides during the digestion process. Conversely, the elongation of F-actin has been measured with a dye-labelled G-actin probe [46]. In this case, the anisotropy is enhanced as a function of time owing to the increasing polymerization of actin.

Time-resolved

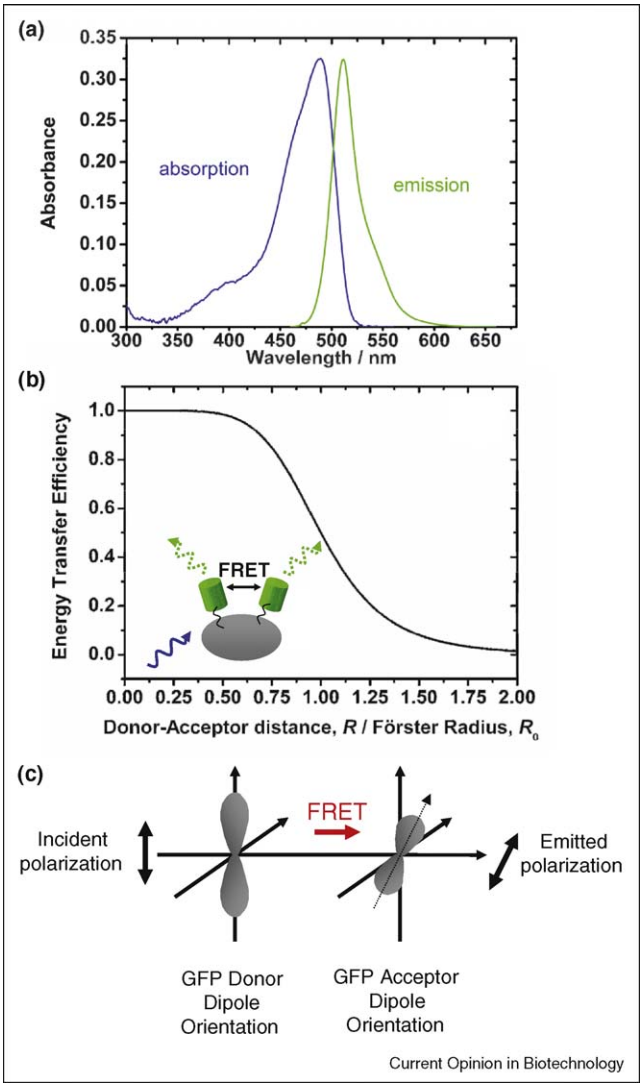
While steady-state anisotropy imaging with polarization-resolved beam-splitting optics is relatively simple, the added complexity of implementing time-resolved fluorescence anisotropy imaging and analyzing the data has meant that there are relatively few examples to date. While time-resolved fluorescence anisotropy decay curves have been measured in cells through microscopes, images where the contrast is generated by the rotational correlation time are rare. A wide-field, time-gated system with a polarization-resolved imager and intensified CCD

detection was used to measure the effects of solvent viscosity on the rotational correlation time of rhodamine 6G [47] and also live B-cells stained with a fluorescein derivative [48]. In practice, measurements of the parallel and perpendicularly polarized components of the time-resolved fluorescence can be made over a whole image or by selecting a region of interest or single 'spot' and recording anisotropy decays. The latter allows for better statistics in the anisotropy decays with typically reduced acquisition times but sacrifices imaging the spatial variations in the anisotropy explicitly. Wide-field, frequency-domain anisotropy FLIM (termed rFLIM) was used to measure anisotropy decays of dyes in solutions of varying viscosity and GFP in bacteria [49], and a system based on wide-field TCSPC for anisotropy measurements has recently been presented [50]. An example of polarization-resolved FLIM of a GFP-tagged KIR receptor in a natural killer cell is shown in Figure 3, using the experimental set-up shown in Figure 1. The rotational correlation time of GFP is around 15 ns, in good agreement with previous work and the value expected for a 27 kDa protein. In a study of IgE receptor cross-linking, FLIM and one-photon and two-photon steady-state fluorescence anisotropy imaging was complemented by time-resolved anisotropy measurements on small defined regions of interest. Using dye-labelled IgE receptor complexes nanoscale membrane order was probed in mast cells [51]. It was proposed that the cross-linking resulted in ordered domains that act to facilitate signalling. Polarization-difference imaging and time-resolved anisotropy have also been used to study cancerous tissues. It was shown that Cybesin-stained cancerous tissue exhibits a higher anisotropy value than normal tissue [52], and this may have potential as a diagnostic tool for distinguishing cancerous and healthy tissue in clinical screening.

Hetero-FRET

Measurements using fluorescent proteins may be complicated by the fact that the rotational correlation time is long compared to the fluorescence lifetime, in contrast to smaller dye molecules, for example rhodamine 6G, where the opposite is true and a complete anisotropy decay can be recorded [19]. However, the slow rotational diffusion times for fluorescent proteins can be advantageous for polarization-resolved microscopy measurements of FRET because of a potentially higher dynamic range than that afforded by FLIM. The method has been applied to image COS7 cells expressing variants of the fluorescent proteins Venus and Cerulean as a FRET pair both in a wide-field and laser scanning configuration. The study showed the viability of polarization-resolved imaging as an indicator of FRET with potential for high throughput screening assays [53]. Hetero-FRET using CFP and YFP measured via anisotropy was further used to show conformational changes in a human G-protein-coupled receptor. The changes resulted from applied shear stress and mechano-chemical modifications of the

Figure 2



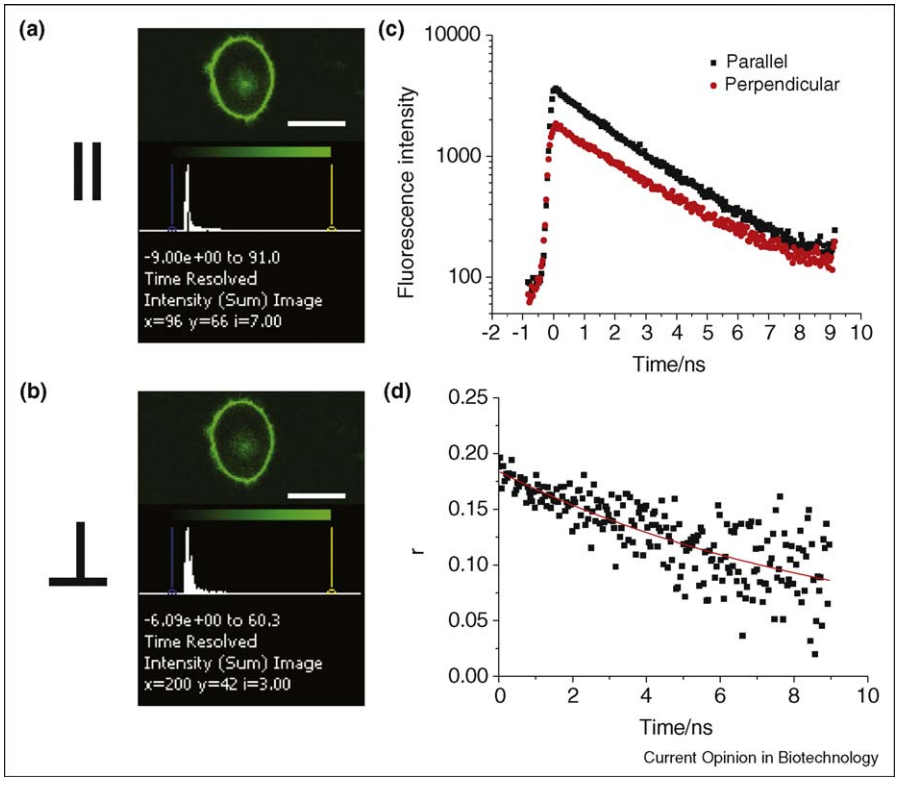
GFP photophysical properties and homo-FRET. **(a)** The absorption and emission spectra of GFP. The small Stokes shift means that there is a significant overlap between the spectra. **(b)** The energy transfer efficiency vs. distance separating the fluorophores for FRET. When the donor–acceptor distance, R, is equal to the Förster radius, R_0, the transfer efficiency is 50%. The cartoon (inset) shows the case for a system with two GFP labels (either double-labelled or dimerized) undergoing homo-FRET. Upon excitation, energy transfer occurs in both directions (i.e. the GFP is both donor and acceptor). Both of the GFP labels emit and as such no shortening of the GFP fluorescence lifetime is observed, nor is there a change in the emission spectrum. The phenomenon can only be observed using polarization-resolved measurements as the occurrence of homo-FRET leads to a decrease in fluorescence anisotropy. **(c)** A schematic representation of the depolarization process during homo-FRET. Molecules with transition dipole moments for absorption oriented parallel to the polarization state of the incident light are preferentially excited. If FRET occurs then energy is transferred non-radiatively to an acceptor molecule whose transition dipole moment for emission may not be parallel to that of the donor molecule. In this case the emitted radiation is depolarized, and for a system where both donor and acceptor are GFP, measuring the polarization state of the emission is the only method for determining whether FRET has occurred.

physical properties of the plasma membrane of endothelial cells [54]. The findings may aid identifying the role of mechanical perturbations in signalling events.

Homo-FRET

The requirements for FRET to occur include sufficient overlap of the emission and absorption spectra of the donor and acceptor molecules respectively. In the case where the conditions for FRET are met by a pair of like chromophores with a small Stokes shift, homogeneous transfer, also known as energy migration FRET (emFRET) or homo-FRET, can occur. This phenomenon is not typically manifested in a shortening of the donor lifetime, a change in the steady-state intensity or a shift in the emission spectrum, as the donor and acceptor are essentially chemically and spectroscopically identical (Figure 2a). However, it has recently been shown that homo-FRET in certain specific fluorescent protein constructs does affect the measured fluorescence lifetime [55]. In general, the non-radiative transfer of energy between identical fluorophores introduces an additional depolarization pathway for the fluorescence that can be detected using polarization-resolved measurements (Figure 2b and c). An intrinsic benefit of homo-FRET over hetero-FRET is that the bioengineering or other labelling technique requires only a single type of fluorescent probe, and it is particularly useful for oligomerization studies. Moreover, the use of a single fluorophore allows for a larger spectral window to be available for other spectral measurements within the same sample. The presence of homo-FRET depolarization, as opposed to rotational depolarization, can be exposed spectrally by excitation on the red-edge of the absorption band of a fluorophore. This acts to decrease the overlap of the absorption and emission bands, reducing the extent of energy transfer, and therefore of the consequent depolarization. The technique has been presented for GFP dimers and trimers in live cell anisotropy imaging [56]. Wide-field, frequency-domain rFLIM measurements were able to show the occurrence of homo-FRET in GFP-expressing *E. coli* bacteria [49]. Caution should also be exercised when performing FRET measurements using CFP or Cerulean with extended time periods for illumination due to distortions in the decays as a function of illumination time [57]. Homo-FRET offers a unique way of identifying oligomerization of labelled molecules

Figure 3



Example of polarization-resolved fluorescence imaging and anisotropy decays from GFP-labelled KIR receptor in a natural killer cell. The data were measured using a laser scanning confocal microscope. The sample was excited using linearly polarized light from a Ti:Sapphire laser with orthogonal polarization components of the emitted fluorescence collected simultaneously using two photomultipliers and TCSPC detection. Intensity images of **(a)** parallel and **(b)** perpendicularly polarized fluorescence emission (relative to the incident light) with **(c)** corresponding time-resolved fluorescence decay curves extracted by binning pixels in the images. After applying Eq. (3), **(d)** an anisotropy decay for the region of interest is extracted. The red line is a fit to the time-resolved GFP fluorescence anisotropy decay curve, yielding a rotational correlation time of 15 ns. The scale bar on the images is 10 μm.

in biological environments, as it is sensitive to the separation of fluorophores on a nanometer scale. Typically, oligomerization of macromolecules should result in an increase of the rotational correlation time, so a decrease in the measured anisotropy is strongly indicative of the competing occurrence of homo-FRET in the sample. Homo-FRET coupled with hetero-FRET and theoretical modelling showed the presence of nanometer-sized clusters of GPI-anchored proteins at the plasma membrane of cells [58]. Similarly, homo-FRET measurements using a time-gated photon counting confocal anisotropy imaging system showed 3D clustering of GFP-labelled GPI in the plasma membrane of NIH 3T3 fibroblasts with the size of the clusters being evaluated using photobleaching methods [59••]. Moreover, in a non-imaging modality homo-FRET measured via fluorescence anisotropy has provided information regarding nanometer-scale heterogeneity in lipid-order in the plasma membrane of cells [61]. In another recent development to aid quantitative data analysis, a theoretical treatment for extracting the oligomer size and degree of oligomerization has been presented, including the concentration dependence of labelled species and distributions of oligomerization states [60••].

Conclusion and outlook

FLIM continues to gain favour as a powerful technique for biological sciences, and in particular determination of protein interactions via FRET and applications to high throughput screening and drug discovery. Technological advances in excitation sources, detectors, data processing and analysis will ensure that it becomes more accessible for researchers. By exploiting the characteristics of fluorescence using multi-parameter methods in an imaging modality, more information can be gained about the sample in a single measurement, and the applicability of the technique will become more widespread. In particular, the extra information afforded by polarization-resolved methods for determination of the chemical and physical environments of macromolecules in intracellular environments seems yet not to have been fully exploited. The extra dynamic range available for hetero-FRET measurements along with the capability of measuring oligomerization via homo-FRET offer distinct advantages over spectrum or lifetime-only alternatives. Multidimensional measurements can be made using fairly simple additions to existing FLIM apparatus, making available a wealth of information from fluorescence on a single, robust experimental platform. Combination of polarization-resolved fluorescence lifetime imaging with total internal reflection microscopy will also afford advantages owing to the polarization properties of evanescent waves [62]. Ultimately, incorporation of multi-parameter detection, reduced acquisition times and rapid data processing will allow for a more reliable understanding of protein interaction and cell dynamics across the biological and life sciences.

Note added in proof

Since submission of the manuscript, a review of time-resolved fluorescence anisotropy has appeared [65], as well as a book chapter on FRET detection by polarization-resolved fluorescence microscopy [69]. Moreover, steady state anisotropy imaging of the mitochondria stain DASPMI has been used to elucidate membrane potential in cells, [66] and fluorescence-detected linear dichroism imaging has been used to study membrane ruffles at the periphery of an intercellular contact (immune synapse) between an natural killer cell and a target cell [67]. Recent detector development with relevance to FLIM has also been described: a 128×128 single photon avalanche diode (SPAD) array with 32 independent timing circuits and picosecond time resolution has been designed, manufactured and used for laser detection and range finding [68]. With a 320 MHz count rate capability, this SPAD array will be very useful for FLIM, as the authors point out [68].

Acknowledgements

We would like to thank Prof Dan Davis and Anne Chauveau in the Division of Cell and Molecular Biology, Imperial College London, for cells and discussions. We would also like to thank the UK Biotechnology and Biological Sciences Research Council (BBSRC) for funding. We are also most grateful to Dr Robert Dale of the Randall Division for Cell and Molecular Biophysics at King's College London for valuable discussions and critical reading of the manuscript, and to Dr Paul Barber from the Gray Institute of Radiation Oncology and Biology, University of Oxford, for the TRI2 FLIM analysis software [70].

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Giepmans BNG, Adams SR, Ellisman MH, Tsien RY: **Review—the fluorescent toolbox for assessing protein location and function**. *Science* 2006, **312**:217-224.

2. Resch-Genger U, Grabolle M, Cavaliere-Jaricot S, Nitschke R, Nann T: **Quantum dots versus organic dyes as fluorescent labels**. *Nat Methods* 2008, **5**:763-775.

3. Festy F, Ameer-Beg SM, Ng T, Suhling K: **Imaging proteins *in vivo* using fluorescence lifetime microscopy**. *Mol Biosystems* 2007, **3**:381-391.

4. •• Verveer PJ, Bastiaens PIH: **Quantitative microscopy and systems biology: seeing the whole picture**. *Histochem Cell Biol* 2008, **130**:833-843.
An excellent contextualization not only of FLIM and fluorescence imaging methods but also of protein–protein interactions. This includes the role mathematical modelling must play to unravel the complex interaction and signalling network in living cells, that is, systems biology.

5. Hille C, Berg M, Bressel L, Munzke D, Primus P, Löhmannsröben HG, Dosche C: **Time-domain fluorescence lifetime imaging for intracellular pH sensing in living tissues**. *Anal Bioanal Chem* 2008, **391**:1871-1879.

6. Nakabayashi T, Wang HP, Kinjo M, Ohta N: **Application of fluorescence lifetime imaging of enhanced green fluorescent protein to intracellular pH measurements**. *Photochem Photobiol Sci* 2008, **7**:668-670.

7. van Manen HJ, Verkuijlen P, Wittendorp P, Subramaniam V, van den Berg TK, Roos D, Otto C: **Refractive index sensing of green fluorescent proteins in living cells using fluorescence lifetime imaging microscopy**. *Biophys J* 2008, **94**:L67-L69.

8. Tregidgo C, Levitt JA, Suhling K: **Effect of refractive index on the fluorescence lifetime of green fluorescent protein**. *J Biomed Opt* 2008, **13**:031218.

9. Kuimova MK, Yahioglu G, Levitt JA, Suhling K: **Molecular rotor measures viscosity of live cells via fluorescence lifetime imaging**. *J Am Chem Soc* 2008, **130**:6672-6673.

10. De Beule PAA, Dunsby C, Galletly NP, Stamp GW, Chu AC, Anand U, Anand P, Benham CD, Naylor A, French PMW: **A hyperspectral fluorescence lifetime probe for skin cancer diagnosis**. *Rev Sci Instrum* 2007, **78**:123101.

11. Galletly NP, McGinty J, Dunsby C, Teixeira F, Requejo-Isidro J, Munro I, Elson DS, Neil MAA, Chu AC, French PMW *et al.*: **Fluorescence lifetime imaging distinguishes basal cell carcinoma from surrounding uninvolved skin**. *Br J Dermatol* 2008, **159**:152-161.

12. Jares-Erijman EA, Jovin TM: **Imaging molecular interactions in living cells by FRET microscopy**. *Curr Opin Chem Biol* 2006, **10**:409-416.

13. Padilla-Parra S, Auduge N, Coppey-Moisan M, Tramier M: **Quantitative FRET analysis by fast acquisition time domain FLIM at high spatial resolution in living cells**. *Biophys J* 2008, **95**:2976-2988.

14. Domingo B, Sabariegos R, Picazo F, Llopis J: **Imaging FRET standards by steady-state fluorescence and lifetime methods**. *Microsc Res Tech* 2007, **70**:1010-1021.

15. Rizzo MA, Springer G, Segawa K, Zipfel WR, Piston DW: **Optimization of pairings and detection conditions for measurement of FRET between cyan and yellow fluorescent proteins**. *Microsc Microanal* 2006, **12**:238-254.

16. Kennedy GT, Elson DS, Hares JD, Munro I, Poher V, French PMW, Neil MAA: **Fluorescence lifetime imaging using light emitting diodes**. *J Phys D—Appl Phys* 2008, **41**:094012.

17. Elder AD, Frank JH, Swartling J, Dai X, Kaminski CF: **Calibration of a wide-field frequency-domain fluorescence lifetime microscopy system using light emitting diodes as light sources**. *J Microsc—Oxford* 2006, **224**:166-180.

18. • Esposito A, Gerritsen HC, Wouters FS: **Optimizing frequency-domain fluorescence lifetime sensing for high-throughput applications: photon economy and acquisition speed**. *J Opt Soc Am A—Opt Image Sci Vis* 2007, **24**:3261-3273.
A thorough study of the efficiency of photon detection in FLIM, with particular focus on the frequency domain approach.

19. Tramier M, Coppey-Moisan M: **Fluorescence anisotropy imaging microscopy for homo-FRET in living cells**. In *Fluorescent Proteins,* edn 2. Edited by Sullivan KF. **Methods in Cell Biology**. 2008:395-414.

20. Kumar S, Dunsby C, De Beule PAA, Owen DM, Anand U, Lanigan PMP, Benninger RKP, Davis DM, Neil MAA, Anand P *et al.*: **Multifocal multiphoton excitation and time correlated single photon counting detection for 3-D fluorescence lifetime imaging**. *Opt Expr* 2007, **15**:12548-12561.

21. van Munster EB, Goedhart J, Kremers GJ, Manders EMM, Gadella TWJ: **Combination of a spinning disc confocal unit with frequency-domain fluorescence lifetime imaging microscopy**. *Cytometry Part A* 2007, **71A**:207-214.

22. Grant DM, McGinty J, McGhee EJ, Bunney TD, Owen DM, Talbot CB, Zhang W, Kumar S, Munro I, Lanigan PMP *et al.*: **High speed optically sectioned fluorescence lifetime imaging permits study of live cell signaling events**. *Opt Expr* 2007, **15**:15656-15673.

23. Esposito A, Dohm CP, Bahr M, Wouters FS: **Unsupervised fluorescence lifetime imaging microscopy for high content and high throughput screening**. *Mol Cell Proteomics* 2007, **6**:1446-1454.

24. Rück A, Hulshoff C, Kinzler I, Becker W, Steiner R: **SLIM: a new method for molecular imaging**. *Microsc Res Tech* 2007, **70**:485-492.

25. Knemeyer JP, Marme N, Hoheisel JD: **Spectrally resolved fluorescence lifetime imaging microscopy (SFLIM)—an appropriate method for imaging single molecules in living cells**. *Anal Bioanal Chem* 2007, **387**:37-40.

26. De Beule P, Owen DM, Manning HB, Talbot CB, Requejo-Isidro J, Dunsby C, McGinty J, Benninger RKP, Elson DS, Munro I *et al.*: **Rapid hyperspectral fluorescence lifetime imaging**. *Microsc Res Tech* 2007, **70**:481-484.

27. Becker W, Bergmann A, Biskup C: **Multispectral fluorescence lifetime imaging by TCSPC**. *Microsc Res Tech* 2007, **70**:403-409.

28. Liu L, Qu J, Lin Z, Wang L, Fu Z, Guo B, Niu H: **Simultaneous time- and spectrum-resolved multifocal multiphoton microscopy**. *Appl Phys B—Lasers Opt* 2006, **84**:379-383.

29. Qu J, Liu L, Chen D, Lin Z, Xu G, Guo B, Niu H: **Temporally and spectrally resolved sampling imaging with a specially designed streak camera**. *Opt Lett* 2006, **31**:368-370.

30. Ahmed Z, Schüller AC, Suhling K, Tregidgo C, Ladbury JE: **Extracellular point mutations in FGFR2 elicit unexpected changes in intracellular signalling**. *Biochem J* 2008, **413**:37-49.

31. Auksorius E, Boruah BR, Dunsby C, Lanigan PMP, Kennedy G, Neil MAA, French PMW: **Stimulated emission depletion microscopy with a supercontinuum source and fluorescence lifetime imaging**. *Opt Lett* 2008, **33**:113-115.

32. Elder AD, Matthews SM, Swartling J, Yunus K, Frank JH, Brennan CM, Fisher AC, Kaminski CF: **The application of frequency-domain fluorescence lifetime imaging microscopy as a quantitative analytical tool for microfluidic devices**. *Opt Expr* 2006, **14**:5456-5467.

33. Robinson T, Valluri P, Manning HB, Owen DM, Munro I, Talbot CB, Dunsby C, Eccleston JF, Baldwin GS, Neil MAA *et al.*: **Three-dimensional molecular mapping in a microfluidic mixing device using fluorescence lifetime imaging**. *Opt Lett* 2008, **33**:1887-1889.

34. Treanor B, Lanigan PMP, Kumar S, Dunsby C, Munro I, Auksorius E, Culley FJ, Purbhoo MA, Phillips D, Neil MAA *et al.*: **Microclusters of inhibitory killer immunoglobulin like receptor signaling at natural killer cell immunological synapses**. *J Cell Biol* 2006, **174**:153-161.

35. Parsons M, Messent AJ, Humphries JD, Deakin NO, Humphries MJ: **Quantification of integrin receptor agonism by fluorescence lifetime imaging**. *J Cell Sci* 2008, **121**:265-271.

36. Jose M, Nair DK, Reissner C, Hartig R, Zuschratter W: **Photophysics of clomeleon by FLIM: discriminating excited state reactions along neuronal development**. *Biophys J* 2007, **92**:2237-2254.

37. Jose M, Nair DK, Altrock WD, Dresbach T, Gundelfinger ED, Zuschratter W: **Investigating interactions mediated by the presynaptic protein bassoon in living cells by Foerster's resonance energy transfer and fluorescence lifetime imaging microscopy**. *Biophys J* 2008, **94**:1483-1496.

38. Kinoshita K, Goryo K, Takada M, Tomokuni Y, Aso T, Okuda H, Shuin T, Fukumura H, Sogawa K: **Ternary complex formation of pVHL, elongin B and elongin C visualized in living cells by a fluorescence resonance energy transfer-fluorescence lifetime imaging microscopy technique**. *FEBS J* 2007, **274**:5567-5575.

39. Ganesan S, Ameer-Beg SM, Ng T, Vojnovic B, Wouters FS: **A dark yellow fluorescent protein (YFP)-based resonance energy-accepting chromoprotein (REACh) for Förster resonance energy transfer with GFP**. *Proc Natl Acad Sci U S A* 2006, **103**:4089-4094.

40. • Grant DM, Zhang W, McGhee EJ, Bunney TD, Talbot CB, Kumar S, Munro I, Dunsby C, Neil MAA, Katan M *et al.*: **Multiplexed FRET to image multiple signaling events in live cells**. *Biophys J* 2008:L69-L71.
A convincing demonstration of an experimental set-up that allows imaging of two FRET pairs simultaneously in a single cell.

41. Lakowicz JR: *Principles of Fluorescence Spectroscopy*. edn 3. New York: Springer; 2006.

42. Birch DJS: **Multiphoton excited fluorescence spectroscopy of biomolecular systems**. *Spectrochimica Acta Part A—Mol Biomol Spectrosc* 2001, **57**:2313-2336.

43. Rao J, Bliattacharya D, Banerjee B, Sarin A, Shivashankar GV: **Trichostatin-A induces differential changes in histone protein dynamics and expression in HeLa cells**. *Biochem Biophys Res Commun* 2007, **363**:263-268.

44. Li W, Wang Y, Shao HR, He YH, Ma H: **Probing rotation dynamics of biomolecules using polarization based fluorescence microscopy**. *Microsc Res Tech* 2007, **70**:390-395.

45. Cao ZH, Huang CC, Tan WH: **Nuclease resistance of telomere-like oligonucleotides monitored in live cells by fluorescence anisotropy imaging**. *Anal Chem* 2006, **78**:1478-1484.

46. Spitz JA, Polard V, Maksimenko A, Subra F, Baratti-Elbaz C, Meallet-Renault R, Pansu RB, Tauc P, Auclair C: **Assessment of cellular actin dynamics by measurement of fluorescence anisotropy**. *Anal Biochem* 2007, **367**:95-103.

47. Siegel J, Suhling K, Lévêque-Fort S, Webb SED, Davis DM, Phillips D, Sabharwal Y, French PMW: **Wide-field time-resolved fluorescence anisotropy imaging (TR-FAIM): imaging the rotational mobility of a fluorophore**. *Rev Sci Instruments* 2003, **74**:182-192.

48. Suhling K, Siegel J, Lanigan PMP, Lévêque-Fort S, Webb SED, Phillips D, Davis DM, French PMW: **Time-resolved fluorescence anisotropy imaging applied to live cells**. *Opt Lett* 2004, **29**:584-586.

49. Clayton AHA, Hanley QS, Arndt-Jovin DJ, Subramaniam V, Jovin TM: **Dynamic fluorescence anisotropy imaging microscopy in the frequency domain (rFLIM)**. *Biophys J* 2002, **83**:1631-1649.

50. Spitz JA, Yasukuni R, Sandeau N, Takano M, Vachon JJ, Meallet-Renault R, Pansu RB: **Scanning-less wide-field single-photon counting device for fluorescence intensity, lifetime and time-resolved anisotropy imaging microscopy**. *J Microsc—Oxford* 2008, **229**:104-114.

51. Davey AM, Walvick RP, Liu YX, Heikal AA, Sheets ED: **Membrane order and molecular dynamics associated with IgE receptor cross-linking in mast cells**. *Biophys J* 2007, **92**:343-355.

52. Pu Y, Wang WB, Achilefu S, Das BB, Tang GC, Sriramoju V, Alfano RR: **Time-resolved fluorescence polarization anisotropy and optical imaging of Cybesin in cancerous and normal prostate tissues**. *Opt Communications* 2007, **274**:260-267.

53. Rizzo MA, Piston DW: **High-contrast imaging of fluorescent protein FRET by fluorescence polarization microscopy**. *Biophys J* 2005, **88**:L14-L16.

54. Chachisvilis M, Zhang YL, Frangos JA: **G protein-coupled receptors sense fluid shear stress in endothelial cells**. *Proc Natl Acad Sci U S A* 2006, **103**:15463-15468.

55. Koushik SV, Vogel SS: **Energy migration alters the fluorescence lifetime of Cerulean: implications for fluorescence lifetime imaging Förster resonance energy transfer measurements**. *J Biomed Opt* 2008, **13**:031204.

56. Squire A, Verveer PJ, Rocks O, Bastiaens PIH: **Red-edge anisotropy microscopy enables dynamic imaging of homo-FRET between green fluorescent proteins in cells**. *J Struct Biol* 2004, **147**:62-69.

57. Hoffmann B, Zimmer T, Klöcker N, Kelbauskas L, König K, Benndorf K, Biskup C: **Prolonged irradiation of enhanced cyan fluorescent protein or Cerulean can invalidate Förster resonance energy transfer measurements**. *J Biomed Opt* 2008, **13**:031205.

58. Sharma P, Varma R, Sarasij RC, Ira, Gousset K, Krishnamoorthy G, Rao M, Mayor S: **Nanoscale organization of multiple GPI-anchored proteins in living cell membranes**. *Cell* 2004, **116**:577-589.

59. •• Bader AN, Hofman EG, Henegouwen P, Gerritsen HC: **Imaging of protein cluster sizes by means of confocal time-gated fluorescence anisotropy microscopy**. *Opt Expr* 2007, **15**:6934-6945.
An excellent paper detailing the use of time-resolved fluorescence anisotropy microscopy to elucidate the numbers of fluorophores in clusters of the lipid marker GPI labelled with GFP. The authors report on small clusters in the plasma membrane of NIH 3T3 cells.

60. •• Yeow EKL, Clayton AHA: **Enumeration of oligomerization states of membrane proteins in living cells by homo-FRET spectroscopy and microscopy: Theory and application**. *Biophys J* 2007, **92**:3098-3104.
The authors present a theoretical description of the nature of homo-FRET between fluorophores in various oligomerization states. A discussion of the concentration dependence of homo-FRET is followed by specific examples from the literature including GFP-tagged proteins.

61. Sengupta P, Holowka D, Baird B: **Fluorescence resonance energy transfer between lipid probes detects nanoscopic heterogeneity in the plasma membrane of live cells**. *Biophys J* 2007, **92**:3564-3574.

62. Smith T, Gee ML, Scholes CA: **Time-resolved evanescent wave-induced fluorescence anisotropy measurements**. In *Reviews in Fluorescence 2005.* Edited by Geddes CD, Lakowicz JR. New York: Springer; 2005:245-271.

63. Bigelow CE, Conover DL, Foster TH: **Confocal fluorescence spectroscopy and anisotropy imaging system**. *Opt Lett* 2003, **28**:695-697.

64. Bigelow CE, Vishwasrao HD, Frelinger JG, Foster TH: **Imaging enzyme activity with polarization-sensitive confocal fluorescence microscopy**. *J Microsc* 2004, **215**:24-33.

65. Vogel SS, Thaler C, Blank PS, Koushik SV: **chapter 10: Time Resolved Fluorescence Anisotropy**. In *FLIM Microscopy in Biology and Medicine*. Edited by Periasamy A, Clegg RM: Taylor & Francis; in press.

66. Ramadass R, Bereiter-Hahn J: **How DASPMI Reveals Mitochondrial Membrane Potential: Fluorescence Decay Kinetics and Steady-State Anisotropy in Living Cells**. *Biophys J* 2008, **95**:4068-4076.

67. Benninger RKP, Vanherberghen B, Young S, Taner SB, Culley FJ, Schnyder T, Neil MAA, Wüstner D, French PMW, Davis DM, Önfelt B: **Live Cell Linear Dichroism Imaging Reveals Extensive Membrane Ruffling within the Docking Structure of Natural Killer Cell Immune Synapses**. *Biophys J* 2009, **96**:L13-L15.

68. Niclass C, Favi C, Kluter T, Gersbach M, Charbon E: **A 128 $\times$ 128 Single-Photon Image Sensor With Column-Level 10-Bit Time-to-Digital Converter Array**. *IEEE Journal of Solid State Circuits* 2008, **43**:2977-2989.

69. Piston DW, Rizzo MA: **FRET by Fluorescence Polarization Microscopy**. In *Fluorescent Proteins*, edn 2. Edited by Sullivan KF. **Methods in Cell Biology**. 2008:415–430.

70. Barber PR, Ameer-Beg SM, Gilbey J, Carlin LM, Keppler M, Ng TC, Vojnovic B: **Multiphoton time-domain fluorescence lifetime imaging microscopy: practical application to protein–protein interactions using global analysis**. *J. R. Soc. Interface* 2009, **6**:S93-S105.