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Advanced techniques in fluorescence microscopy

by Heiko Gaethje

Fluorescence microscopy is a powerful tool in life science research, and recent years have witnessed a rapid development of techniques and applications, from FRET and FLIM to TIRF and other live cell imaging approaches. These achievements have been driven by the development of new hardware, such as sensitive CCD cameras and new lasers, by the development of application oriented software and the integration of these innovations into dedicated live cell imaging systems and confocal laser scanning microscopes. Hard- and software improvements have been accompanied by the design of new fluorescent probes and labelling chemistries. This article summarises some of the latest trends in fluorescence microscopy applications.

Sequencing the human genome was a milestone on the way to understanding the structure and function of organisms. However, the genetic code of a protein does not reveal its function. For this to be elucidated, genes have to be cloned and the expression patterns and (sub)cellular localisations of the corresponding proteins identified using techniques such as fluorescence *in situ* hybridisation (DNA) or immunofluorescence (protein) on a fixed specimen. The results are snapshots of dynamic processes. To analyse molecular functions under physiological conditions other methods had to be devised to allow specific native proteins to be detected and tracked *in vitro* or *in vivo* (in living organisms, tissues and cells).

Distance dependent techniques

Many cellular functions involve movements and interactions of individual molecules, compartments or vesicles within the nm range. Precise localisation and direct tracking of single molecules is a challenge for light microscopy due to the physical limits of optical resolution. The distance dependent techniques described in this section overcome the resolution limits of ~200 nm, thus advancing into the size range of proteins.

TIRFM

Total Internal Reflection Fluorescence Microscopy (TIRFM) is a distance dependent technique with extremely high z-resolution. TIR is an optical phenomenon. If light is travelling through a medium with a high refractive index

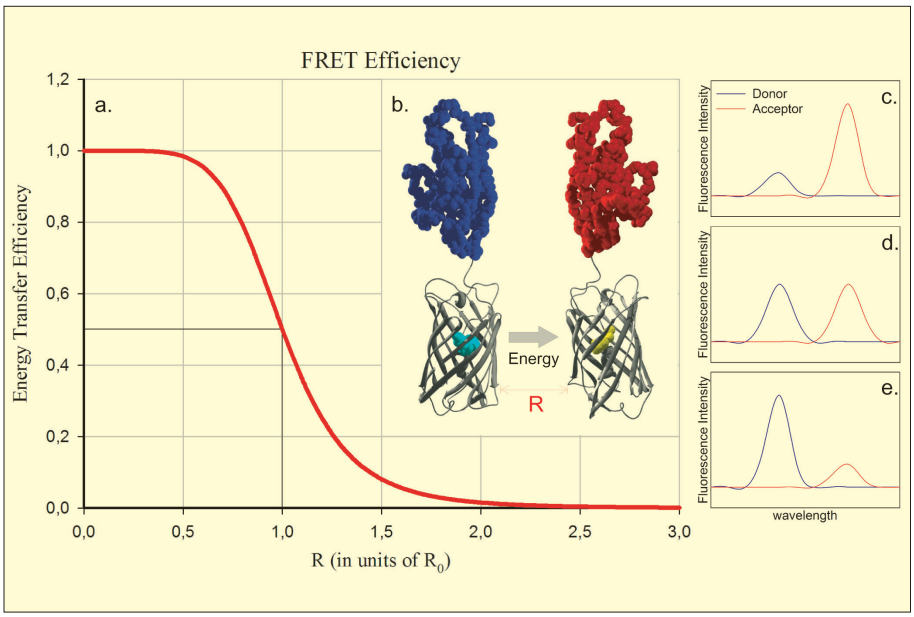

Figure 1. FRET efficiency varies with the inverse sixth power of donor-acceptor fluorophore distance. This dependency results in a fast decaying curve depicted in Figure 1a. The donor-acceptor distance (Figure 1b schematic of the CFP/YFP donor-acceptor pair fused to target proteins) is expressed in units of the Foerster critical distance (R_0), at which the FRET rate equals the donor de-excitation rate via fluorescence emission (marked by the droplines). If the donor and acceptor are in close proximity (<10nm) and illuminated at the donor excitation wavelength, the donor emission is quenched and the absorbed energy is transferred to the acceptor which emits fluorescent light (sensitised emission, Figure 1c). At R_0 one half of the donor molecules are de-excited via FRET and the other half via fluorescence emission (Figure 1d). If no FRET occurs, the donor emission fluorescence is measured (Figure 1e).

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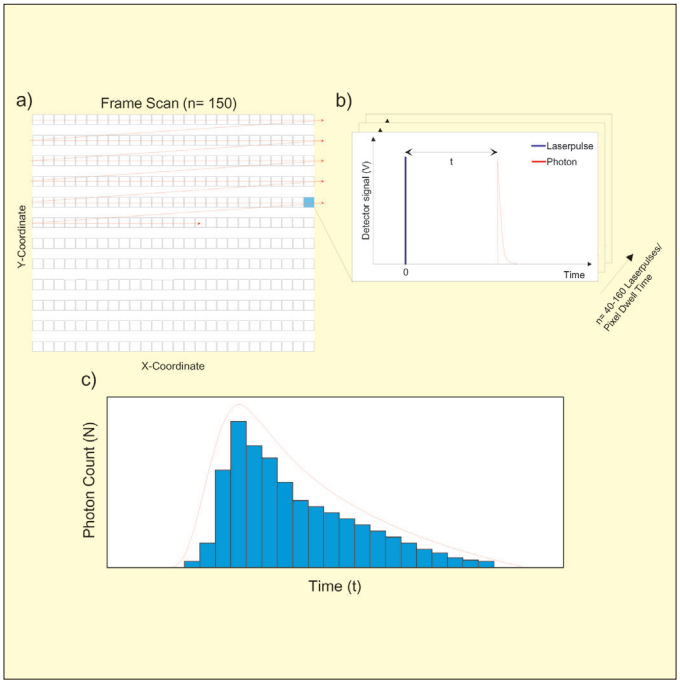

Figure 2. Time correlated single photon counting: The specimen image is acquired line by line and pixel by pixel (Figure 2a) with a laser scanning microscope (e.g. Olympus FV1000). If the pixel dwell time of the confocal laser scanning microscope is 2 µs and the laser is pulsed with 20-80 MHz, a pixel receives 40-160 pulses per frame scan and a maximum number of 40-160 fluorescent photon counts per pixel might be detected. Each counted photon is stored with the XY-coordinates of the pixel and the time between the laser pulse and the detection signal (Figure 2b). These data are accumulated in a histogram for each pixel. By repeating the frame scan process (e.g. n=150), the photon count per pixel is increased to yield meaningful numbers (N 10000-100000) (Figure 2c). The histogram is turned into an exponential decay curve from which the fluorescence lifetime is calculated.

and strikes the interface of an optical medium with a lower refractive index at an angle greater than the critical angle, the incident light will undergo total internal reflection. Under these conditions some light still enters the low refractive index medium as an electromagnetic wave termed the evanescent wave. The intensity of this wave decays exponentially with penetration depth. The average z-expansion is less than 200 nm depending on the wavelength of light, the incident angle and the refractive index of the media. In TIRFM, fluorophores in the sample at a maximum distance of ~100 nm from the coverslip surface are selectively excited. This z-resolution of 100 nm is one-fifth of an optical section obtained with a laser scanning confocal microscope. Therefore TIRFM is ideally suited for the observation of processes and structures on or close to the cell surface. For example, TIRF is employed for the study of cell-substrate boundaries (focal contacts), exocytosis, vesicle and granule dynamics, membrane morphology and submembrane cytoskeletal elements.

A typical TIRF microscope setup consists of an epifluorescence microscope in combination with laser light source(s), special TIRF-illuminators and high numerical aperture objectives (e.g. the Olympus PLAPON60xO-TIRFM [NA 1.45], PLAPO100xO-TIRFM [NA 1.45], APO 100xOHR [NA 1.65]). This company offers individual optical components for TIRFM as well as turnkey solutions based on the Olympus cell^R and cell^M imaging stations.

FRET

Fluorescence- (or Foerster-) Resonance Energy Transfer (FRET) is another distance dependent physical process. The energy absorbed by a donor fluorophore is transferred to an acceptor fluorophore by intermolecular dipole-dipole coupling. FRET efficiency decreases in proportion to the inverse sixth power of increasing intermolecular distance and approaches zero around 10 nm [Figures 1a and 1b]. Thus FRET is ideally suited for investigating molecular distances as well as stable and dynamic molecular interactions in the range of 1-10 nm. Donor-acceptor pairs for FRET have to fulfil certain requirements: the excitation spectra of both fluorophores must be separated sufficiently to allow for selective stimulation; donor emission and acceptor excitation spectra should overlap by at least 30% for efficient FRET; and the emission spectra of the FRET-pair should be well separated to facilitate distinct measurement of the respective fluorescence intensities.

In practice FRET can be qualitatively or quantitatively detected using intensity measurements of the fluorescence emission. The ratio between the measured intensity of the sensitised acceptor emission and the intensity of the donor emission in the FRET sample [Figures 1c, 1d and 1e] is calculated. The ratio is also determined for corresponding reference samples without FRET. For quantitative measurements the concentrations of fluorescently labelled molecules must be tightly controlled and spectral deconvolution approaches are used to remove artefacts resulting from insufficient separation of donor-acceptor excitation and emission spectra. GFP and its homologues are widely used donor-acceptor pairs [Figure 1b] because DNA recombination techniques allow intracellular expression of target molecules directly coupled to these markers.

FRET has been successfully applied to examine protein folding/unfolding and the conformational changes of proteins. In addition, the assembly and spatial composition of protein complexes as well as receptor/ligand interactions have been

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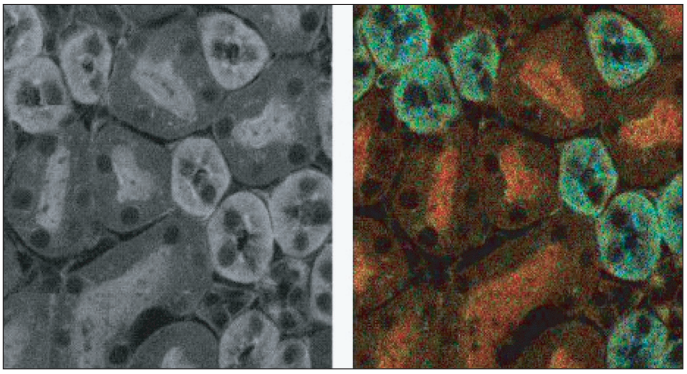

Figure 3. Fluorescence images of stained mouse kidney (Molecular Probes). Left: intensity image of the laser scanning microscope. Right: FLIM image. Blue: lifetime 0.8 ns, green: lifetime 2.1 ns, red: lifetime 4.5 ns). Data courtesy of Uwe Ortmann, Picoquant GmbH, Berlin, Germany.

followed using this technique. It is also possible to study membrane fluidity, distribution, the transport of lipids, membrane fusion and to perform membrane potential measurements with FRET.

To measure FRET in living cells an inverse epifluorescence microscope equipped with specific filter sets, a sensitive digital camera and image analysis software are necessary. Wide-field illumination is usually sufficient. To obtain images without out-of-focus blur, laser scanning confocal-FRET with tuneable lasers is applied, which provides the additional advantage of 3D image acquisition. The cell^R imaging station (wide-field) and the FluoView1000 confocal laser scanning microscope are optimally suited for FRET experiments.

BRET

Bioluminescent Resonance Energy Transfer (BRET) is based on the same physical principle as FRET. The difference between this technique and FRET is that luciferase is used as the donor molecule. This bioluminescent protein eliminates the need for fluorescence excitation. As a consequence, bleedthrough and photobleaching artefacts are avoided. Bioluminescence light is transferred to a GFP-acceptor.

Time-resolved techniques

FLIM

Fluorescence multi-colour staining experiments simultaneously visualise and distinguish different molecules in a cell which may interact or co-localise. Typically the spectral characteristics of the respective fluorophores are utilised for differentiation. However, the number of fluorophores that can be differentiated simultaneously without significant artefacts is limited to 5 or 6 due to spectral overlaps.

This limitation does not apply for a technique called Fluorescence Lifetime Imaging Microscopy (FLIM). The fluorescence lifetime is the average period of time (in nanoseconds/picoseconds) an excited fluorophore remains in the high energy state before it returns to the basic energy level by emitting fluorescence. The fluorescence lifetime is very specific for each fluorophore. By measuring differences in fluorescence lifetime it is possible to distinguish dyes with almost identical emission spectra. Another potential application of FLIM is the acquisition of images with high signal-to-noise ratios using fluorophores with longer lifetimes compared to the unspecific specimen autofluorescence.

The fluorescence lifetime is influenced by many environmental parameters. FLIM is therefore not only used for multi-colour staining, but also utilised to measure changes in means of energy transfer (FRET). The combination of FRET and FLIM overcomes many of the problems associated with intensity-based FRET measurements because the fluorescence lifetime is not affected by fluorophore concentration, photobleaching, light scattering and excitation light intensity.

There are two principal methods for fluorescence lifetime imaging: the time-domain method and the frequency-domain method. In the frequency-domain method the fluorophores are excited by sinusoidal modulated laser light and the fluorescence lifetime is calculated from the phase shift of the emission signal. In the time-domain technique, Time-Correlated Single Photon Counting (TCSPC) is used for FLIM in conjunction with the Olympus FV1000 upgraded with Picoquant equipment [Figure 2]. The fluorochrome is excited periodically by a laser pulse originating from a pulsed laser diode or femtosecond pulsed Titanium:Sapphire laser. A photomultiplier or single photon avalanche diode detects the photons emitted from the fluorophore. The time between the laser pulse and the detection of a photon is measured. A histogram accumulates the photons corresponding to both the X-Y image position and the relative time between the laser pulse and the detection signal. Every pixel of the FLIM image contains the information of a complete fluorescence decay curve. If an image is composed out of three fluorochromes with different lifetimes, the distribution of all dyes can be shown in false colours [Figure 3].

FRAP/FLIP

Two fluorescence microscopy techniques are available for examining active movement or diffusion of intracellular or membrane bound molecules: Fluorescence Loss In Photobleaching (FLIP) and Fluorescence Recovery After Photobleaching (FRAP). Typically a specific area of a cell membrane previously labelled with a floating fluorescent dye is bleached with a confocal laser scanning microscope and the

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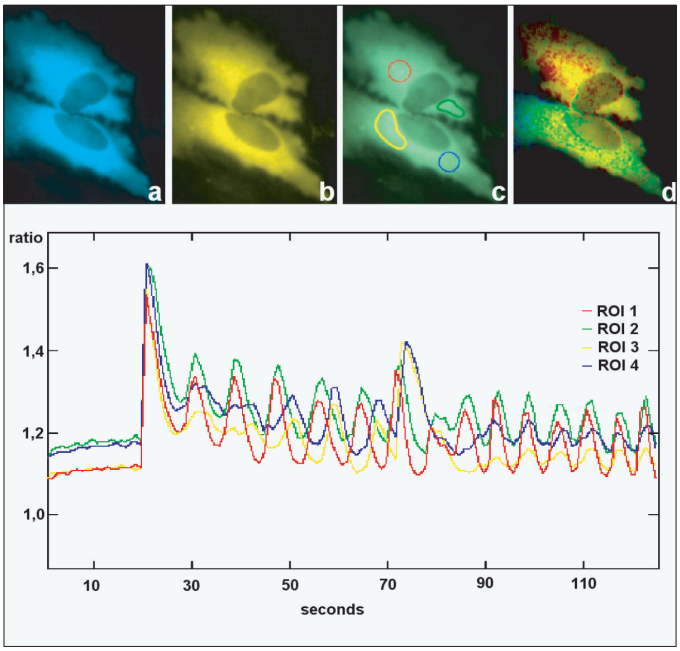

Figure 4. HeLa cell labelled with CFP/YFP Cameleon, triggered with histamine. a: CFP channel, b: YFP channel, c: CFP/YFP double emission image acquired with a Dual-View Micro-Imager, d: ratio image. The graphical analysis shows the ratio changes in the ROIs revealing FRET effects caused by a change of Ca^{2+} concentration. Data courtesy of H. Mizuno, A, Miyawaki, Brain Science Institute, RIKEN, Wako, Saitama, Japan.

loss or recovery of fluorescence over time is recorded to determine the lateral membrane fluidity. In addition, FLIP is useful for verifying the continuity of membranous organelles. If a small, circumscribed area of an organelle is continuously bleached, the fluorescence from the non-illuminated part will slowly vanish if unbleached fluorophores can laterally diffuse into the illuminated spot. This will continue until all the fluorescent molecules are depleted.

FCS

Fluorescence Correlation Spectroscopy (FCS) is a confocal technique used to measure molecular dynamics in highly diluted sample solutions. Researchers have used this technique to gain information about chemical reaction rates, flow rates, diffusion coefficients, molecular weights and molecular aggregates. A collimated laser beam is focussed through a high numerical aperture objective to irradiate a cone shaped area of the sample solution. A volume of approximately one femtolitre ($1\mu m^3$) is precisely in focus and this comprises the measurement region. Fluorescent signals from this area enter the objective, pass the confocal optics and are detected, while out of focus blur is blocked.

Intensity fluctuations arising from the number or quantum yield of the few fluorescently labelled target molecules diffusing in and out of the measurement volume are recorded over time. Small molecules diffuse rapidly in random paths through the volume, generating short, randomised bursts of intensity. Larger molecular complexes move more slowly and produce a longer, more sustained time-dependent fluorescence intensity pattern. The mean diffusion time, which is affected by the molecular weight, can be calculated on the basis of the temporal signal correlations of many single molecules.

Ion-measurements

Signal transduction processes often involve transient and rapid changes in ion concentrations. Calcium concentration changes are common signals for various cellular events, e.g. muscle contraction. Fluorescent calcium indicators, such as Fura-2, Fluo-3 or Calcium Green, show differences in fluorescence lifetime and intensity upon calcium binding. By measuring the fluorescence intensity over time, the ratio of dyes in the calcium bound and unbound state can be determined, indicating concentration changes. FLIM measurements are preferable to measure precise ion concentrations, since fluorescence lifetime is independent of the relative chromophore concentrations which facilitates measurement calibration.

The Cameleon calcium probe is based on the FRET process. Cameleon is a chimeric fusion protein consisting of CFP, Calmodulin, the M13 peptide (Calmodulin binding domain of myosin light chain kinase M13) and YFP. When no calcium is bound, the molecule is stretched out. In this conformation CFP and YFP, which reside on the ends of the molecule, are too far apart for FRET. Calcium binding results in a conformational change that brings CFP and YFP into close proximity (<10 nm), thus facilitating FRET and permitting sensitised fluorescence emission. In summary, ion measurements can be performed using intensity based ratio measurements, FLIM or FRET. The technique chosen depends on the purpose of the measurement, the ion and the fluorescent probe.

The author

Heiko Gaethje
Web Content Manager, Microscopy
Olympus Life and Material Science Europa GmbH
Wendenstraße 14-18
20097 Hamburg
Germany
Email: heiko.gaethje@olympus-europa.com