Image Anal Stereol 2006;25:111-120
Review Article

QUANTITATIVE CONFOCAL LASER SCANNING MICROSCOPY

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(Accepted September 20, 2006)

ABSTRACT

This paper discusses recent advances in confocal laser scanning microscopy (CLSM) for imaging of 3D structure as well as quantitative characterization of biomolecular interactions and diffusion behaviour by means of one- and two-photon excitation. The use of CLSM for improved stereological length estimation in thick (up to 0.5 mm) tissue is proposed. The techniques of FRET (Fluorescence Resonance Energy Transfer), FLIM (Fluorescence Lifetime Imaging Microscopy), FCS (Fluorescence Correlation Spectroscopy) and FRAP (Fluorescence Recovery After Photobleaching) are introduced and their applicability for quantitative imaging of biomolecular (co-)localization and trafficking in live cells described. The advantage of two-photon versus one-photon excitation in relation to these techniques is discussed.

Keywords: CLSM, FCS, FLIM, fluorescence, FRET, sampling, stereology, two-photon excitation.

INTRODUCTION

In recent years, a dramatic development in software, computer power, lasers and detection systems has allowed a wealth of new fluorescence microscopy techniques to be developed that facilitate the quantification of biomolecular diffusion, conformations and interactions in living cells with hitherto unforeseen spatial- and temporal resolution. Examples of such techniques are FRET and FCS by which it is possible to detect a physical interaction between two or more biomolecules on a sub-10 nm scale. In addition, with the new CLSM's, cellular or molecular structure and distribution in living or fixed tissue can be imaged with a spatial resolution close to the theoretical limit. This is because efficient short-pulse lasers are now available for two-photon excitation which facilitates more background-free imaging and deeper penetration into tissue. This means that the field of depth in two-photon imaging is the smallest possible which can be advantageous with some special stereological estimators. This is especially true for some of the virtual test systems on which the randomization of the stereological probes is made within volume probes of arbitrarily orientated thick uniform, random physical sections. Complete isotropy and 2D uniform, random sampling in the thick section of the virtual test system is performed by a computer. Implementation of new virtual test systems may be possible by the use of a CLSM on fluorescently labeled samples.

Fluorescent labels (fluorophores) can be used as markers to obtain information about the localization or trafficking of different kinds of biomolecules within the sample. In traditional fluorescence microscopy the imaging resolution is restricted by the wavelength of light (the so-called diffraction limit). This means that if two different biomolecules (*e.g.* proteins) are observed as being co-localized (*i.e.*, residing within the same pixel on different imaging channels), in reality they can be separated by up to ~500 nm (depending on the laser wavelength used). This limitation is circumvented with the technique of FRET which exploits the principle of a correlated, distance-dependent modulation in the fluorescence emission from two spectrally overlapping fluorophores (a donor and an acceptor) that are situated within a few nanometers from each other. FCS is another technique that is capable of detecting molecular interactions by means of correlated intensity fluctuations of two spectrally distinct fluorophores diffusing within the laser focus. With FRET it is possible to quantify the inter- and intramolecular distances whereas FCS allows for the quantification of diffusion behaviour as well as stoichiometries and rate constants for molecular interactions. Diffusion constants can also be determined with FRAP, which in addition allows for quantification of the fraction of

immobile sub-populations. FLIM is a particular fluorescence detection mode that allows the lifetimes of the fluorophores to be measured in addition to the intensities that are normally detected. In the latter case it is possible to discriminate between different fluorophores with similar spectral characteristics if their lifetimes are sufficiently well separated. This is particularly convenient for cellular imaging with green fluorescent protein (GFP) which has a spectrum that exhibits a significant overlap with the autofluorescence present in most cell types.

Of the two basic imaging modes available, wide-field and confocal, the latter provides for the largest degree of versatility and highest spatial resolution and we will limit ourselves to a discussion of this type of microscopy. The concept of a confocal microscope is shown in Fig. 1: a point-like spot of illumination is scanned across a plane to create an image onto a pinhole that is confocal with the illumination point (Valeur, 2002). Since the fluorescence signal from above and below the plane of the pinhole is blocked, a much better depth resolution (optical sectioning) is obtained as compared with wide-field imaging. Today, a state-of-the-art confocal laser-scanning microscope system is equipped with continuous-wave (CW) lasers of various different colors typically spanning the range of 405-633 nm which gives access to the one-photon absorption spectra of most available fluorophores. In addition, a short-pulsed laser can be added for two-photon excitation. On the detection side the microscope is equipped with detectors for spectral scanning and can be equipped with acquisition- and analysis software for FRET, FLIM, FCS and FRAP.

METHODS

TWO-PHOTON MICROSCOPY

Two-photon microscopy is an optical sectioning technique that uses ultrashort-pulsed (~100 femto-seconds = $100 \cdot 10^{-15}$ s) infra-red light to excite fluorescent probes usually excited by longer-pulsed (≥100 picoseconds = $100 \cdot 10^{-12}$ s) or CW ultraviolet- or visible light (So *et al.*, 2000; Zipfel *et al.*, 2003; Rubart, 2004). Since the probability of simultaneous absorption of two photons is proportional to the square of the laser intensity (Patterson and Piston, 2000), excitation is restricted to a very tiny volume in the sample (~0.1 femtoliter). It follows that any fluorescence detected originates from that volume only, allowing extremely efficient direct detection of all the signal (including

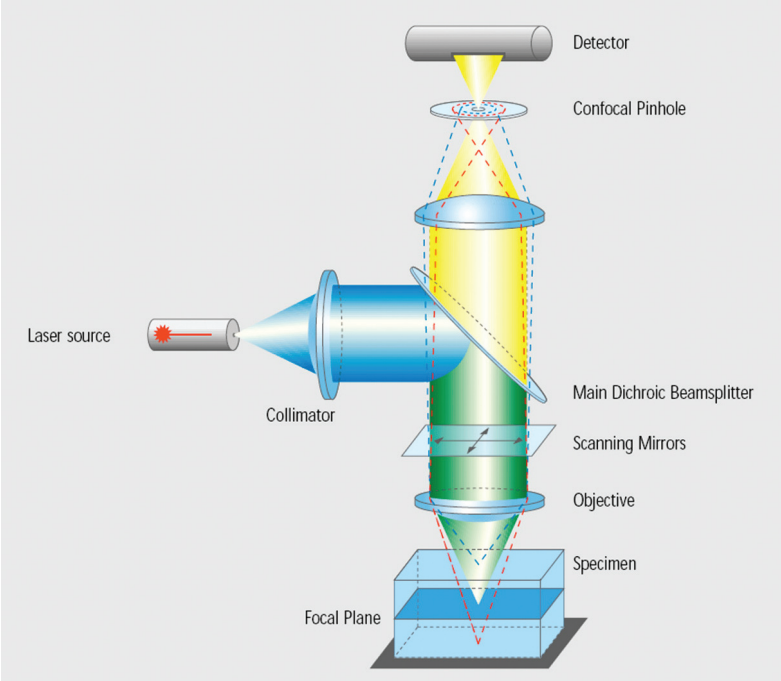

Fig. 1. *The principle of a confocal microscopy setup. Light from a laser point source is reflected off a dichroic mirror and imaged onto the specimen. Only fluorescence light emitted from the focal plane is detected since out-of-focus light is unable to pass through the pinhole. Reproduced with permission from Simbürger et al (2006).*

scattered light) without any need for confocal pin holes. Thus, optical sectioning with two-photon imaging is intrinsic to the excitation process. The axial resolution is in principle similar to that obtained with one-photon excitation using properly adjusted pin holes but in the one-photon case fluorophores residing above and below the focal spot are to a much larger extent excited. This is because of the linear dependence on intensity of the probability for one-photon absorption (Rubart, 2004). This will give rise to out-of-focus fluorescence which, for relatively thick specimens (tissue sections), will be scattered into the detection volume and blur the image.

Since infrared light is scattered to a much lesser extent than visible and UV wavelengths, it penetrates at least two-fold deeper into tissue (~500 µm) and thus allows for 3D imaging of thicker specimens (Rubart, 2004). In addition, with shorter pulses the same level of intensity during the presence of light can be achieved with much reduced average laser power compared to continuous-wave illumination, minimizing heat dissipation and consequently cellular damage. Although two-photon excitation might give rise to a slight increase in photobleaching within the focal volume compared to one-photon excitation, this is compensated by virtually no photobleaching outside of this volume. The advantage of one- vs. two-photon excitation in terms of photobleaching is still a matter of debate, *see e.g.* (Patterson and Piston, 2000; Eggeling *et al.*, 2005). What is significant to a first approximation is not the (peak) intensity (energy/area/time which is orders of magnitudes higher for femtosecond pulses compared to continuous-wave illumination) but the fluence (energy/area delivered within a certain time period), which is not significantly different in either case.

Fig. 2 shows the energy-level diagram of the electronic ground state as well as an excited electronic state for a molecule (*e.g.*, a fluorophore) with the one- and two-photon excitation transitions indicated together with the fluorescence emission. The combined energy of two low-energy photons is equivalent to the energy of one high-energy photon and the one- and two-photon fluorescence spectra look the same. Due to quantum mechanical selection rules it is, however, not the same excited state which is populated as reflected in a difference in the absorption spectra; experimentally determined two-photon absorption spectra often have a different shape and the maximum is shifted with respect to their one-photon counterparts. As a consequence, it is sometimes possible to find pairs of spectrally well separated fluorophores which can be two-photon excited with the same infrared wavelength. This is an advantage in dual-channel FCS but a disadvantage in FRET.

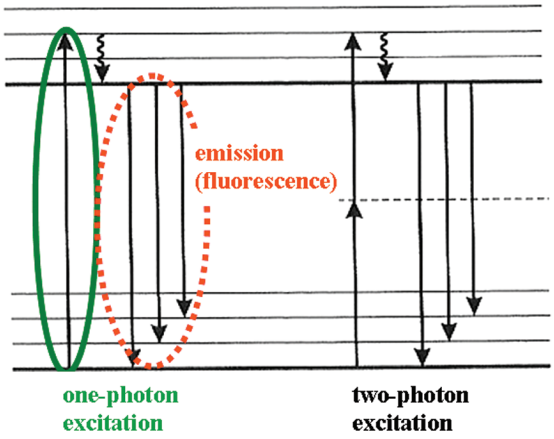

Fig. 2. *Molecular energy - level diagram showing one- and two-photon excitation from the ground state to an excited electronic state. For each electronic state there are a number of vibrational levels providing fast relaxation to the lowest vibrational level of the ground- or excited electronic state.*

The shorter the laser pulse the less power is required to reach the appropriate intensity (~50 GW/cm^2) (Patterson and Piston, 2000) for two-photon excitation and the less heat is dissipated in the sample due to a lower average power. This favours the use of femto-second lasers as opposed to the cheaper picosecond lasers. More importantly, although picosecond lasers are available in different colours they are not tunable; in contrast femtosecond lasers (based on Ti: Sapphire) can be tuned over 700-1000 nm with sufficient output power to cover the two-photon excitation spectra of most available fluorophores. Currently, lasers with pulse widths down to 10 fs are commercially available but a pulse width below 100 fs is not practically feasible. This is because significant pulse broadening and reshaping is encountered when guiding <50 femtosecond pulses through the optics of the microscope, requiring the use of extended optical (chirp) compensation.

STEREOLOGICAL VIRTUAL TEST SYSTEMS

During the last decade, stereology has seen great advances in the use of virtual test systems. The novel concept of spatial isotropic uniform random sampling with virtual test systems in thick (>25 µm) sections

with an arbitrary orientation may be applied to all stereological estimators. The computer supplies the necessary isotropy of the test systems and the intersection between the focal plane, and the virtual test system is visible and moving when focusing up and down in the thick section. Subsequent sampling is performed by superimposing a computer-generated 2D representation of the test system onto live images of thin focal planes within the uniform, random volume test system. The 2D representation of the test system moves across the computer screen during focal plane displacement and dynamically maps the test system that exists in 3D within the volume probe. Test systems based on isotropic lines in 3D are visualized as moving points in the 2D focal plane (Kubínová and Janáček, 1998), and test systems based on isotropic planes in 3D are visualized as moving lines (Larsen *et al*., 1998).

The design of virtual test systems may in principle only be limited by computer power and our imagination. Test systems that are isotropic in 3D allow for sampling inside a thick, arbitrarily orientated uniform, random physical section, thereby reducing considerably the problems associated with estimation under anisotropic conditions. The major practical advantage of this principle is that for most orientation-dependent estimators there is complete freedom to choose the most convenient sectioning direction. The challenge is to develop these estimators further into local spatial sampling of volume of uniformly sampled cells: the spatial rotator (JO Larsen, private communication). This is a design-based estimator of number-weighted mean cell volume in thick sections of arbitrary orientation. It allows empirical estimates of the real precision of the estimator and therefore all other estimators of cell volume. There are problems in using the new principle of spatial isotropic uniformly random sampling with virtual probes. Images of the focal plane from thick sections contain out-of-focus information, which reduces the quality of the image. These problems arise from the effects of over- and underprojection of cell borders in optical sections inside thick physical sections and these effects are very difficult to control and to calibrate (Tandrup *et al.*, 1997). The hope is that a Ti:Sapphire femtosecond laser can produce a field of depth which is so thin that the effects of over- and underprojection will be negligible.

FRET (FLUORESCENCE RESONANCE ENERGY TRANSFER)

The ability to image specific biomolecules interact within a biological specimen is giving new insights in fundamental cellular processes. Interacting proteins assembling into signaling or transport complexes or other functional units controlling cellular life and death have traditionally been studied using biophysical or biochemical methods such as affinity chromatography or co-immunoprecipitation. While these in vitro screening methods can be used to elucidate unknown protein partners, they do not however, allow direct access to interactions of these protein partners in the living cell. Using the approach of FRET microscopy, this information can be obtained from single living cells with nanometer resolution (*see* Fig. 3) (Lippincott-Schwartz *et al.*, 2001; Zhang *et al.*, 2002). FRET occurs when two fluorophores 1) are in close proximity (1-10 nm); 2) have a favorable dipole-dipole orientation, as well as 3) a sufficiently large spectral overlap.

FRET is a process by which electronic excitation energy is transferred from a laser-excited (green) donor fluorophore to a spectrally red-shifted acceptor fluorophore (or quencher), both of which are attached to a single biomolecule or two different interacting biomolecules. Upon energy transfer the donor fluorophore is quenched and the fluorescence from the acceptor fluorophore increases. The probability E of energy transfer is related to the distance R between the donor and acceptor fluorophores according to (Lakowicz, 1999; Enderlein, 2003):

$$E = \frac{1}{1+\left(R/R_0\right)^6}$$

where

$$R_0 = \frac{9Q_D\kappa^2}{8\pi(2\pi)^4 n^4}\frac{\int d\lambda F_D \varepsilon_A(\lambda)\lambda^4}{\int d\lambda F_D(\lambda)}. \qquad (1)$$

R_0 is the so-called Förster distance which is around 5 nm for a typical donor-acceptor pair. When the fluorophores are separated by this distance the probability of energy transfer will be 50% and the technique is very sensitive to distance changes around this value as illustrated in Fig. 4, showing E as a function of R for a donor-acceptor pair with $R_0 = 5$ nm.

In this respect FRET microscopy is highly superior to conventional optical microscopy for which the spatial resolution is limited by the wavelength of light. In the formula for R_0, Q_D and F_D are the quantum yield and fluorescence signal from the donor, ε_A the extinction coefficient of the acceptor at wavelength λ, and n is the refractive index of the medium while κ is a factor expressing the orientation between the donor and acceptor dipoles. The integral in the numerator is expressing the degree of spectral overlap of donor emission with acceptor absorption. Usually the spectral overlap can be considered constant apart from special cases where the donor or acceptor becomes chmically modulated (as in *e.g.*, a redox reaction, *see* Schmauder *et al.*, 2005).

From the above formula for E the distance R can be determined if E can be measured and R_0 assumed to be known. R_0 can be difficult to determine with accuracy, though, since Q_D is sensitive to the environment of the donor and the value to be used for n is also not always obvious (Knox, 2002). There are different ways in which E can be determined, for example from the difference in intensity of the donor fluorescence signal or lifetime depending on an acceptor being present or not (Chen *et al.*, 2003; Enderlein, 2003; Zal *et al.*, 2004):

$$E = \frac{I_D - I_{DA}}{I_D} = \frac{\tau_D - \tau_{DA}}{\tau_{DA}}. \qquad (2)$$

Here, I_D and I_{DA} are the donor intensities in the absence and presence of acceptor and τ_D and τ_{DA} the corresponding donor lifetimes. The presence of an acceptor is signified as a decrease in the donor fluorescence intensity and lifetime. It should be noted that the E value stated above applies to a single donor-acceptor pair. In a real sample with unequal amounts of donors and acceptors the measured value E_{app} must be corrected for the ratio $[DA]/[D_{total}]$ of donor-acceptor complexes $[DA]$ to total number of donors $[D_{total}]$ (Zal *et al.*, 2004). This, however, only applies to the intensity-based method since lifetimes are independent of concentration. In addition, if the difference in lifetime of the donor with and without acceptor quenching is sufficiently large , the lifetimes can be distinguished in a single measurement and there is no need for control samples or acceptor photobleaching as required in the intensity-based measurements. Lifetimes can, however, only be measured with an expensive FLIM-based system, requiring, *e.g.*, a pulsed laser and a specialized computer interface card plus software as described in more detail in the following section.

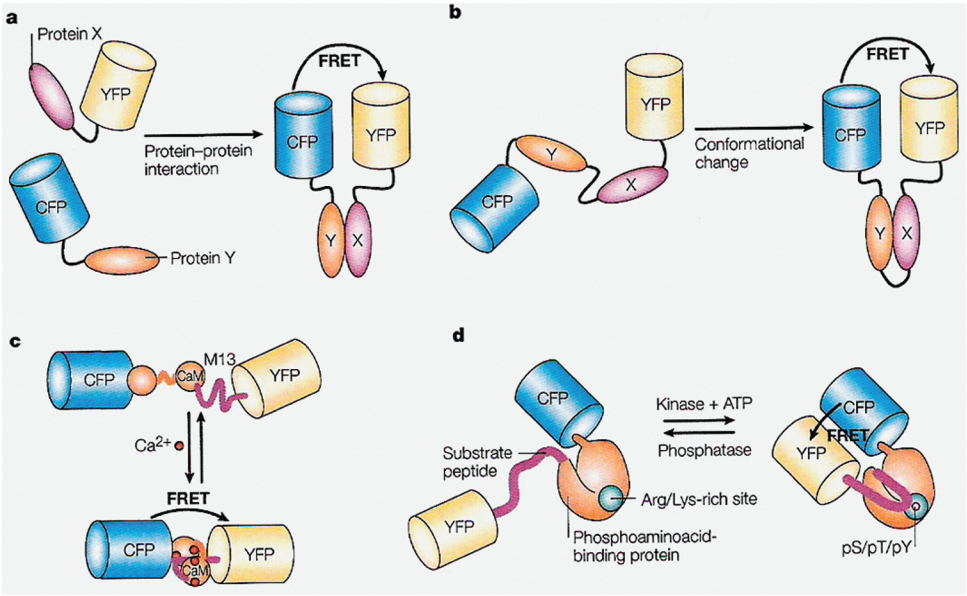

Fig. 3. *Application of FRET for monitoring (a) protein-protein interactions, (b) conformational changes within a protein, (c) the presence of Ca^{2+} and (d) protein phosphorylation. The principles are illustrated using cyan and yellow fluorescent proteins (CFP and YFP) as donors and acceptors, respectively. X and Y denote two different types of proteins (a) and two different domains within the same protein, respectively. In (c) the presence of Ca^{2+} is detected by means of a fusion protein consisting of a pepide domain M13 recognizing calmodulin bound to Ca^{2+}. In (d) a substrate peptide is recognized by a specific S, T or Y site of a phosphoaminoacid-binding protein upon its phosphorylation (Arg, arginine; Lys, lysine; pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine). Reprinted with permission from Zhang et al (2002).*

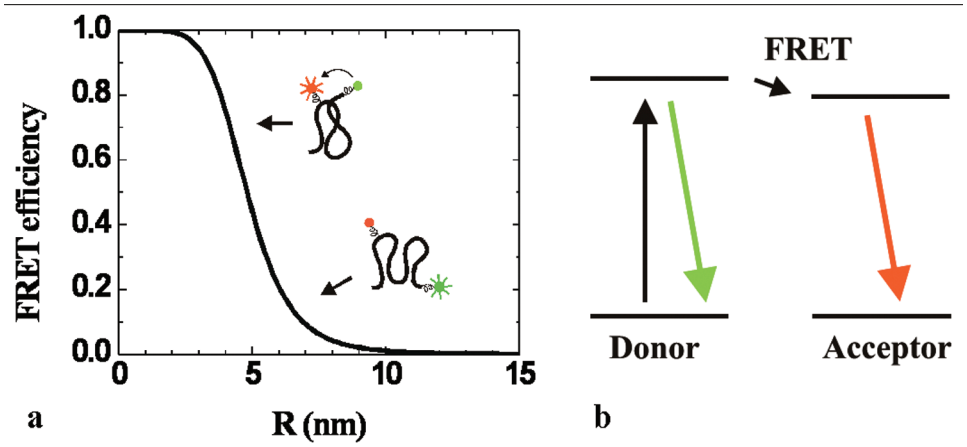

Fig. 4. *(a) FRET efficiency as a function of donor-acceptor distance for a FRET pair with R_0 = 5 nm and (b) sketch of the electronic energy level diagram of donor and acceptor; the transfer of excitation energy is indicated with a black arrow, and the donor and acceptor fluorescence emissions with green and red arrows, respectively.*

By labeling the proteins of interest with appropriate FRET pairs, protein- protein interactions can be elucidated directly in living or fixed cells by studying the transfer of energy between an excited donor fluorophore and a non-excited acceptor fluorophore. Fluorophores could be fluorescent proteins (*e.g.*, CFP, YFP) fused to *e.g.* receptors or ligands of interest (expressed in the cell) or to specific antibodies recognized by these proteins. Alternatively, antibodies or ligands can be labeled covalently with small organic chromophores (*e.g.*, Alexa or Cy dyes). Although most studies so far have been concerned with living cells with only a couple of results published on fixed cells (tissue) (Chen *et al.*, 2003; Mills *et al.*, 2003) there is in principle nothing which restricts this method to living cells. It is, however, most beneficial for looking at biomolecular interactions within specific cellular compartments and is not really useful for sampling large segments of tissue. FRET can be performed with one- as well as two-photon excitation with pulsed as well as continuous-wave excitation. Pulsed excitation facilitates the use of FLIM with two-photon excitation in particular providing for the benefits described in previously.

FLIM (FLUORESCENCE LIFETIME IMAGING MICROSCOPY)

Spectral bleedthrough of (especially) the donor signal into the acceptor channel can be a problem in FRET but can be avoided by the application of FLIM (Periasamy, 2001). The fluorophore is not only characterized by its emission spectrum but also by the time it remains in the excited stated before returning to the ground state - its fluorescence lifetime. The lifetime of the fluorophore is critically dependent on its environment and this gives advantages in imaging dynamic cellular events (Borst *et al.*, 2005; Suhling *et al.*, 2005). FLIM detects the nanosecond decay of the donor fluorophore and therefore provides an alternative way of measuring FRET since the lifetime of the donor fluorophore is directly related to the donor-acceptor distance as explained in the previous section. Fluorescence lifetimes can be measured at each point in the image and are sensitive to changes in the microenvironment such as pH, ion concentration, etc., but not to the concentration of the fluorophore (Martin-Fernandez *et al.*, 2004; Suhling *et al.*, 2005). While most commercially available confocal microscopes are able to do spectral scanning, FLIM is technically somewhat more challenging in terms of computer card technology and detector requirements and has so far only been offered by specialized companies. Combined solutions are now starting to become commercially available but with the FLIM detection system to be considered more as an add-on than a fully integrated module. FLIM will provide a complementary and possibly more error-free way of doing FRET than the intensity-based methods most frequently applied (Zal *et al.*, 2004; Chen and Periasamy, 2006).

FLIM can be performed in two different ways; in the frequency or time domain. In the frequency domain method a continuous-wave light source and the fluorescence detector are modulated periodically and from the phase shift or so-called amplitude modulation ratio of the fluorescence signal the lifetime can be deduced (van Geest and Stoop, 2003).

This method is mostly suited for a wide-field system with a CCD camera and will not be discussed further here. It has the advantage that no scanning is required but deducing more than one lifetime component requires an acquisition with a number of different modulation frequencies and extended deconvolution procedures. In the time-domain approach a pulsed (femtosecond) laser with ~100 MHz repetition rate is used for excitation and a point detector (photo-multiplier tube) with a fast response time applied for fluorescence detection. It is based on the concept of time-correlated single-photon counting (TCSPC) where the time delay from a laser pulse hits the sample until a fluorescence photon hits the detector is measured for a series of laser pulses over a time period of ~1 s, depending on the signal level and number of lifetime components present in the fluorescence decay (Becker *et al.*, 2004).

The concept of TCSPC relies on a time-to-amplitude converter which acts as a timer that is trigged (or started) every time a laser pulse hits the sample and stopped every time a photon hits the detector (Valeur, 2002). The corresponding time delay is converted into a voltage which is read by a computer card that functions as a multichannel analyzer, generating a histogram of number of photon events detected as a function of the time delay. This gives rise to a lifetime curve which can be fitted to one or more exponentials, depending on the number of lifetime components, to yield the lifetime(s) of the fluorescence decay(s). Since only one stop pulse can be handled per start pulse, the laser intensity must be low enough that the probability of emission of more than one photon per laser pulse is vanishingly small. Therefore, in order to collect as many photons as possible within the shortest possible time period the repetition rate of the laser should be as high as possible but still low enough that the interval between laser pulses is a few times the value of the longest lifetime (which is typically a few nanoseconds). Even under these optimum conditions the acquisition of a FLIM image of a few hundred by a few hundred pixels takes a few minutes (Becker *et al.*, 2004), somewhat longer than an ordinary intensity-based confocal acqusition, which limits the use of FLIM in time-lapse studies.

FCS (FLUORESCENCE CORRELATION SPECTROSCOPY)

FCS and FRAP are concerned with measurements of intracellular diffusion and thus show their strength in living tissue at the sub-cellular level (Haustein and Schwille, 2003; Day and Schaufele, 2005; Schwille and Haustein, 2006). FCS is not restricted to measurements of molecular diffusion but can in principle be used to measure the diffusion time of any object, *i.e.*, also a cell. However, cells are relatively immobile in tissue and in practice measurements are only possible on cells residing in a relative non-viscous environment. With FCS it is in addition possible to detect biomolecular interactions but only if the interacting molecules are diffusing. Furthermore, distances between fluorophores on interacting proteins cannot be quantified. On the other hand, in contrast to FRET there is no strict requirement for a sub-10 nm distance between the fluorophores in order for the interaction to be detected which gives a somewhat larger degree of freedom in design of the biological constructs.

Since FCS essentially has single-molecule sensitivity, it is able to separate two or more populations of biomolecules with different diffusion behavior, provided the diffusion constants of the different species are well enough separated. However, in order to collect enough single-molecule statistics for these parameters to be determined with reasonable accuracy, diffusion must take place on a sub-second time scale and this is why FCS (like FRAP) is not very suitable for fixed cells. With FRAP, fluorescently labeled proteins within a specific subregion of the sample are momentarily bleached and the time it takes for the fluorescent protein signal to recover within this region is monitored. Like FCS, FRAP allows for quantification of diffusion times (provided they are well enough separated) but not diffusion behavior or complex formation. On the other hand, with FRAP it is possible to detect and quantify an eventual immobile fraction of molecules.

FCS measures the intensity fluctuations δI(t) as a function of time caused by minute deviations in *e.g.* fluorophore concentration or photophysical properties of fluorescence-labelled biomolecules diffusing within the focal volume (Haustein and Schwille, 2003; Schwille and Haustein, 2006):

$$\delta I(t) = I(t) - \langle I(t) \rangle_t, \quad \langle I(t) \rangle_t = \frac{1}{T} \int_0^T I(t) dt .$$

The fluorescence intensity fluctuations are analysed with a digital correlator, which builds a temporal auto- or cross-correlation curve. This curve provides information on diffusion rates, local concentrations of molecules and rate constants for protein-protein interactions. For each type of fluorophore the autocorrelation curve can be constructed as sketched in Fig. 5 (*see* Haustein and Schwille, 2003; Schwille and Haustein, 2006) for further details):

$$G(\tau) = \frac{\langle \delta I(t) \cdot \delta I(t+\tau) \rangle_t}{\langle I(t) \rangle_t^2}. \quad (3)$$

Theoretically, the auto-correlation curve is related to the mean number $\langle N \rangle$ of fluorescently labelled molecules in the focal spot and the diffusion time τ_D according to

$$G(\tau) = \frac{1}{\langle N \rangle}\left(1+\frac{\tau}{\tau_D}\right)^{-1}\left(1+\frac{\tau}{\tau_D}\left(\frac{r_0}{z_0}\right)^2\right)^{-1/2} \quad (4)$$

assuming that the intensity fluctuations are due to concentration fluctuations alone. τ_D is related to the diffusion constant D according to $\tau_D = r_0^2/D$ and r_0 and z_0 are characteristic radial and transverse dimensions of the focus. Knowing these dimensions $\langle N \rangle = 1/G(0)$ and D can be determined for a given type of fluorescently labelled biomolecular species by a fit to the theoretical expression, and from $\langle N \rangle$, r_0 and z_0 the mean concentration of molecules can be obtained.

If a mixture of two different types of biomolecules (with two different types of fluorophores) are present FCS can be performed with two different laser colours and the resulting auto-correlation curve is obtained as a sum of the auto-correlation curves for the individual species. Since for lateral diffusion D scales with the mass M of the biomolecule as $D \propto M^{1/3}$ and assuming that the diffusion constants have to differ by a factor two in order to be separated with reasonable accuracy in the fit of this autocorrelation curve, this implies that the mass difference between the two species must differ by at least a factor of 8. This means that in practice molecular interactions and the corresponding change in diffusion constant cannot be determined from a measurement of an auto-correlation curve alone. Instead one can do a cross-correlation since the mean number $\langle N_{12} \rangle$ of complexes of species 1 and 2 can be directly obtained from the ratio of the cross-correlation amplitude $G_x(0)$ to the product of the individual auto-correlation amplitudes $G_1(0)$ and $G_2(0)$ (Haustein and Schwille, 2003; Schwille and Haustein, 2006). FCS with two-photon excitation, currently limited to very few groups, offers the unique possibility of exciting the two different fluorophores with the same laser line, facilitating dual-channel cross-correlation without the problems of alignment and different focal spot sizes that are otherwise complicating the dual-colour analysis. It might be a challenge, however, to find a pair of dyes with sufficient spectral emission separation that can be excited with the same laser intensity without one of them undergoing significant photobleaching.

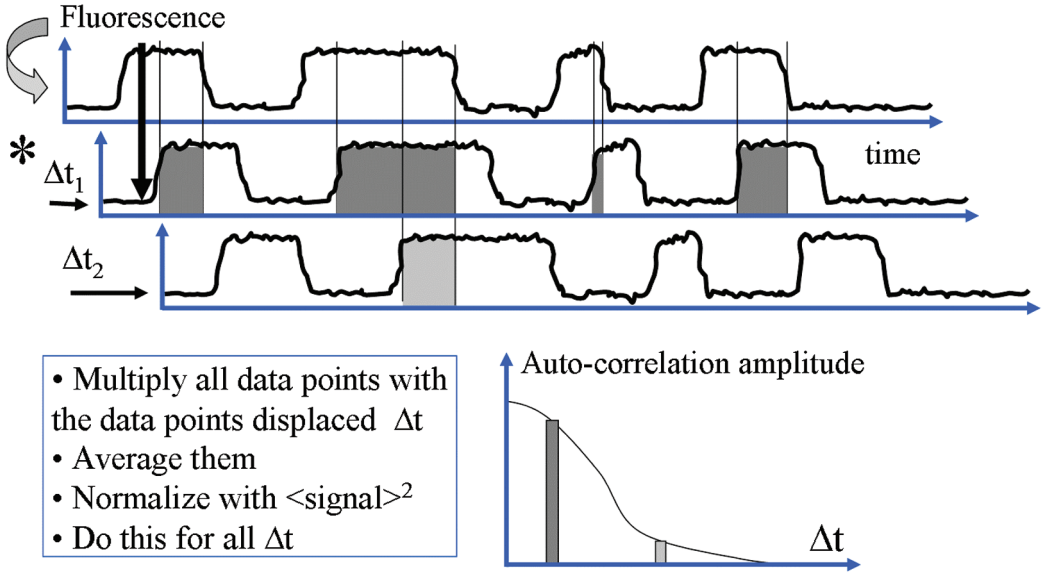

Fig. 5. *Steps involved in the construction of an FCS auto-correlation curve; the fluorescence time trace is displaced by an amount Δt and data points from the two traces are pairwise multiplied to create a new trace which is averaged over all time points and normalized wrt. the squared average of the original signal (<signal>^{2}; averaged over all time points). Shown are two examples with Δt = Δt$_1$ and Δt = Δt$_2$. Reproduced with permission from R. Schmauder.*

FRET VERSUS FCS

Both FRET and FCS can be used for probing of biomolecular interactions but only FRET can yield a quantitative measurement of inter- and intra-molecular distances, as signified by the interactions between donor and acceptor fluorophores attached to interacting proteins or within the same protein. FRET is, however, only sensitive to distances and distance changes on the 5-10 nm scale and thus requires some care in the attachment of fluorophores. This rules out certain kinds of studies, for example in case of a ligand interacting with a trans-membrane receptor that can only be labeled at the cytoplasmic domain. With FCS, such limitations are not present but for interacting protein partners that are not well enough separated in mass (differing by less than a factor of ~8), diffusion constants for receptor-bound vs. free single dye-labeled ligands cannot be separated and binding thus not detected. This can be circumvented by labeling the interacting proteins with two different colors (similarly to FRET) and exploiting a two-channel cross-correlation scheme. Furthermore, with FCS it is possible to measure the diffusion constants for receptor and ligand and for each partner even separate sub-populations of different diffusion behavior, provided they are well enough separated (differing by more than a factor of ~2). If this is not the case, one has to resort to wide-field single-molecule tracking, which is more challenging due to the fast photo-bleaching of presently available fluorophores and problems with separating the traces of individual molecules due to the time lag between images (given by the readout time of the CCD). Since with FCS data must be acquired for several seconds in order to collect enough statistics for an auto- or cross-correlation curve, in effect this makes it a much more time- consuming technique compared with FRET, thus limiting its the potential use in scanning mode.

CONCLUSION

The implementation of confocal one- and two-photon microscopy may have great potential in the further development of stereological virtual test systems as well as in the application of advanced quantitative fluorescence techniques: FRET, FLIM and FCS. Femtosecond lasers used for two-photon excitation have a wide range of tunability and are today much more compact and easy to operate than previously but are unfortunately still very expensive. The advantage of using two-photon excitation for more accurate quantification of 3D biological structure as well as biomolecular interactions and diffusion behaviour is beginning to become appreciated.

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