Optimization of fluorescent proteins for novel quantitative multiparameter microscopy approaches
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Fluorescence resonance energy transfer (FRET) measurement by gradual acceptor photobleaching


Fluorescence resonance energy transfer (FRET) is an extremely effective tool to detect molecular interaction at sub-optical resolutions. One of the techniques to measure FRET is acceptor photobleaching: the increase in donor fluorescence after complete acceptor photobleaching is a measure of the FRET efficiency. However, in wide-field microscopy, complete acceptor photobleaching is difficult due to the low excitation intensities. In addition, the method is sensitive to inadvertent donor bleaching, autofluorescence and bleed through of excitation light. In the method introduced in this paper, donor and acceptor intensities are continuously monitored during acceptor photobleaching. Subsequently, curve fitting is used to determine the FRET efficiency. The method was demonstrated on cameleon (YC2.1), a FRET-based Ca\(^{2+}\) indicator, and on a CFP-YFP fusion protein expressed in HeLa cells. FRET efficiency of cameleon in the presence of 1 mM Ca\(^{2+}\) was 31 ± 3 %. In absence of Ca\(^{2+}\) a FRET efficiency of 15 ± 2 % was found. A FRET efficiency of 28 % was found for the CFP-YFP fusion protein in HeLa cells. Advantages of the method are that it does not require complete acceptor photobleaching, includes correction for spectral cross talk, donor photobleaching and autofluorescence, and is relatively simple to use on a normal wide-field microscope.

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4.1 Introduction

Fluorescence Resonance Energy Transfer (FRET) is a process whereby excited state energy is transferred non-radiatively from an excited donor molecule to an acceptor molecule (Förster, 1948). For FRET to occur a number of conditions need to be fulfilled: 1) the emission spectrum of the donor needs to have sufficient overlap with the absorption spectrum of the acceptor, 2) the emission and absorption dipole moment of donor and acceptor must not be perpendicular, 3) donor and acceptor have to be in close proximity. The amount of FRET is usually expressed as FRET efficiency: the fraction of the photons absorbed by the donor whose energy is transferred to the acceptor. In the case of a single acceptor per donor, FRET efficiency is related to the distance between donor and acceptor according to

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

with E, the FRET efficiency, r the distance between donor and acceptor, and R0, the distance at which the FRET efficiency is 0.5. R0 is typically a few nanometers, depending on the donor and acceptor molecule and their relative orientation. Because of this strong distance dependence, FRET is an ideal way to detect molecular proximity at length scales far below the optical resolution. When FRET occurs, several effects occur (Jares-Erijman & Jovin, 2003): 1) donor fluorescence is quenched, 2) donor excited state lifetime decreases, 3) donor anisotropy increases, and 4) provided the acceptor is itself a fluorochrome, the emission of the acceptor is increased (sensitised emission).

For measurement of FRET in microscopy numerous techniques exist (Jares-Erijman & Jovin, 2003). Basically, they can be divided in techniques that require dedicated instrumentation e.g. fluorescence lifetime imaging microscopy (FLIM) (Gadella et al., 1993; Squire & Bastiaens, 1999; van Munster & Gadella, 2005) or anisotropy microscopy (Lidke et al., 2003; Squire et al., 2004), and methods that can be applied on conventional fluorescence microscopes. The latter ones are usually based on sensitised emission, which can be measured by recording the acceptor fluorescence using donor excitation. Several approaches have been published (Gordon et al., 1998; Hoppe et al., 2002; Nagy et al., 1998; Xia & Liu, 2001; Youvan et al., 1997). What they all have in common is that corrections are applied for spectral cross talk, photobleaching and relative abundances of donor and acceptor. In confocal microscopy, also chromatic aberrations and fluctuations of the excitation power should be taken into account (van Rheenen et al., 2004). In practice, due to the large amounts of corrections, obtaining reliable results can be very complicated (Berney & Danuser, 2003). An alternative method is to use photobleaching, which is comparatively simple to perform on a conventional fluorescence microscope. There are multiple ways in which photobleaching can be used. The donor bleach rate is directly
related to the excited state lifetime, and thus provides a way to detect changes in lifetime, analogue to the way this is monitored by FLIM (Bastiaens & Jovin, 1998; Jovin & Arndt-Jovin, 1989; Jovin & Arndt-Jovin, 1989; Kubitscheck et al., 1993; Young et al., 1994). Alternatively, donor bleaching can used to distinguish sensitised acceptor fluorescence from directly excited acceptor fluorescence, the latter one being independent of donor bleaching (Mekler, 1994). Monitoring the decay of sensitised acceptor emission in response to donor photobleaching it is possible to not only measure the FRET efficiency but also the percentage of donors exhibiting FRET (Clayton et al., 2005). A requirement of this technique is that the acceptor is photostable, and the donor photolabile. A variant is measurement of the photobleaching of the acceptor in response to excitation via FRET (Mekler et al., 1997). Here, the acceptor should be photolabile, and the donor photostable. A more straightforward approach is to use direct acceptor photobleaching, thus frustrating the occurrence of FRET, and monitor the reappearance of the donor fluorescence. Basic principle is to measure donor intensity before and after complete acceptor bleaching thus providing an internal control by eliminating the occurrence of FRET. The increase in donor intensity can be directly related to the FRET efficiency (Chan et al., 2001; Gu et al., 2004; Kenworthy, 2001; Llopis et al., 2000; Mochizuki et al., 2001; Sato et al., 2002; Wouter et al., 1998). Also here, measured intensities should be corrected for cross talk of the acceptor into the donor detection channel (Gu et al., 2004). In these methods, acceptor bleaching has to be complete. This limits the applicability of the method for wide-field microscopy where bleach rates are low because of the relatively low excitation intensity. Also, correction for inadvertent donor bleaching is complicated. Donor bleaching occurs by imaging of the donor itself as well as by excitation cross talk during the acceptor bleaching. The first could be determined by comparing the intensity before and after measurement of an object that was not acceptor photo-bleached. The latter can be determined from the decrease in intensity of a donor-only object, preferably within the same image. The assumption here is that the photobleaching characteristics for the donor-only object are the same as that of the object under investigation, which is something that is not necessarily true, given the fact that the bleach rate itself is affect by the occurrence of FRET. Furthermore, the presence of a background intensity in the donor channel, due to autofluorescence or bleed through of the excitation light, will lead to underestimation of FRET efficiencies, especially when signals are low.

**Gradual acceptor photobleaching**

Despite the advantages of detecting FRET with just two images, a serious shortcoming of this method is that artifacts remain obscured when donor intensity is only measured before and after acceptor photobleaching. Obviously, this can interfere with quantification. Measuring donor intensity as a function of time during the bleach process itself, it is possible to detect both inadvertent donor photobleaching and the presence of a background intensity without the need to do additional measurements. This could also make it possible to perform measurements with only partial acceptor photobleaching. To check the feasibility of this approach we focus on what happens if the acceptor is gradually photobleached and donor and acceptor intensities are monitored during the process. If we
assume mono-exponential bleaching, which has been found to be a good approximation for practical purposes (Chen et al., 1995), the acceptor intensity can be written as:

\[ I_a(t) = I_{a,0} \cdot e^{-t/\tau_a} + I_{a,bg} \tag{2} \]

with \( I_{a,0} \), the acceptor intensity at \( t=0 \), \( \tau_a \), the bleach time for the acceptor, and \( I_{a,bg} \), the invariant contribution of autofluorescence and bleed through of excitation light. Assuming that 1) photobleaching occurs indiscriminately to all acceptor molecules, both bound and unbound, and 2) there is not more than one acceptor per donor molecule present, the average FRET efficiency is directly proportional to the amount of available acceptor molecules. Hence, the average FRET efficiency can be written as:

\[ E(t) = E_0 \cdot e^{-t/\tau_e} \tag{3} \]

with \( E(t) \), the FRET efficiency and \( E_0 \) the FRET efficiency at \( t=0 \). Given the fact that the donor intensity is proportional to \( (1-E) \), we can write, taking into account photobleaching of the donor during measurements:

\[ I_d(t) = I_{d,0} \left( 1 - E_0 \cdot e^{-t/\tau_d} \right) \cdot e^{-t/\tau_d} + I_{d,bg} \tag{4} \]

with \( I_{d,0} \), the donor intensity at \( t=0 \) in the absence of FRET, \( \tau_d \), the donor bleach time, and \( I_{d,bg} \), the invariant contribution of autofluorescence and bleed through of excitation light in the donor channel. \( I_d(t) \) and \( I_a(t) \) are depicted in figure 4.1 for several values of \( E_0 \). Donor bleaching used was twenty times slower than the acceptor bleaching. As can be seen, the donor intensity will typically first show an increase before slowly falling off. At low values of \( E_0 \), the increase becomes barely noticeable.

**Figure 4.1** Simulation depicting the intensity of donor and acceptor as function of time during acceptor photobleaching. In this example donor bleaching is twenty times slower than acceptor bleaching. Both donor and acceptor background intensities are zero.
Cross talk correction
In practice, both donor and acceptor intensities should be corrected for cross talk before analysis. Correction of the donor intensity for acceptor cross talk is more complicated than vice versa: The problem is that there are two contributions: 1) cross talk from directly excited acceptor and 2) cross talk from acceptor excitation by FRET (sensitised emission). The first contribution as well as the donor cross talk in the acceptor channel can be corrected for by linear unmixing (Press et al., 1994; Zimmermann et al., 2003): Basically, donor-only, and acceptor-only objects are imaged using both donor and acceptor filter sets, resulting in four images. From the intensities in these images the amount of spectral cross talk of the donor in the acceptor-channel and vice-versa is determined. Correction of measured intensities is done using linear unmixing. After this, there is still a contribution of cross talk of sensitised acceptor emission:

\[ I_d(t) = I_{d,0} \left[ k - E_0 \cdot e^{-t/T} \right] \cdot e^{-t/T} + I_{d,bg} + I_{a,bg}(t) \]  

with \( I_{d,bg} \) the donor intensity corrected for cross talk of directly excited acceptor and \( I_{a,bg} \) the contribution to the measured donor intensity of cross talk of sensitised acceptor emission. The intensity lost due to energy transfer is dependent on the FRET efficiency and the intensity of the donor, both of which are time-dependent due to the photobleaching of both acceptor and donor. The percentage of this intensity that is eventually emitted in the form of acceptor fluorescence is depended on the quantum efficiency of the acceptor. Only a small percentage of this acceptor fluorescence will be detected in the donor detection channel. The increase in measured donor intensity due to this effect can be written as:

\[ I_{a,bg}(t) = Q_a \cdot c \cdot E_0 \cdot e^{-t/T} \cdot I_{d,0} \cdot e^{-t/T} \]  

with \( Q_a \) the quantum efficiency of the acceptor and \( c \) the ratio of the emitted acceptor fluorescence that falls within the detection bandwidth of the donor channel. \( c \) represents the spectral overlap of the donor detection channel with the emission spectrum of the acceptor relative to the overlap with the emission spectrum of the donor. This can be written as:

\[ c = \frac{\int D_d(\lambda) \cdot F_a(\lambda) \cdot d\lambda}{\int D_a(\lambda) \cdot F_d(\lambda) \cdot d\lambda} \]  

with \( D_d(\lambda) \), the sensitivity of the donor detection channel, and \( F_d(\lambda) \) and \( F_a(\lambda) \) the normalized donor and acceptor fluorescence emission, respectively, as function of the wavelength. It should be emphasized that \( c \) is different from the cross talk parameter determined in the linear unmixing. Contrary to the latter parameter, \( c \) is independent from the acceptor detection channel and from the excitation spectra and excitation wavelength used. Combining equations 5 and 6, we obtain:

\[ I_d(t) = I_{d,0} \left[ k - (1 - Q_a) \cdot E_0 \cdot e^{-t/T} \right] \cdot e^{-t/T} + I_{d,bg} \]
As can be seen by comparing equations 4 and 8, the effect of sensitised emission cross talk only influences the value for $E_0$ that is found. The function itself remains the same. The determination of the cross talk parameters has to be performed once for every donor/acceptor pair and filter set.

**Curve fitting**

After recording donor and acceptor intensities as a function of time during acceptor photobleaching and correction for cross talk with linear unmixing, curve fitting of equation 2 to the measured acceptor intensity can be used to obtain $I_{a,0}$, $I_{a,bg}$, and $\tau_a$. Subsequently, fitting of equation 8 to the measured donor intensity can be used to obtain $I_{d,0}$, $I_{d,bg}$, $\tau_d$, and, most importantly, $E_0$.

**In vitro test measurement**

To test the method in practice, *in vitro* samples of purified cameleon Ca$^{2+}$ indicator were measured (Miyawaki *et al.*, 1997). Cameleon is a fusion protein consisting of a Cyan Fluorescent Protein (CFP), calmodulin, a calmodulin binding domain, and a Yellow Fluorescent Protein (YFP). Binding of Ca$^{2+}$ to calmodulin induces intramolecular binding of the calmodulin binding domain to calmodulin thereby changing the spatial conformation of the protein in such a way that CFP and YFP come in close enough proximity for FRET. Ca$^{2+}$ can thus be used to control the amount of FRET measured. The method is tested by measuring the average FRET efficiency in an environment with a high Ca$^{2+}$ concentration and in the absence of Ca$^{2+}$.

**Test on biological samples**

A fusion protein of CFP and YFP linked via a short linker, will display an inherent amount of FRET due to the close proximity of donor and acceptor. Typically, FRET efficiencies in these types of constructs are between 20 and 35% because the size of the proteins does not allow the actual chromophores, which are located inside a barrel structure, to come closer for an higher efficiency. To test the method introduced in this paper a fusion construct was brought to expression in HeLa cells. After fixation, the acceptor was photobleached and donor and acceptor intensities were monitored during the process to obtain the FRET efficiency.

**4.2 Materials & Methods**

**Preparation of cameleon samples**

For protein isolation, YC2.1, an improved version of cameleon, (Miyawaki *et al.*, 1999), was cut from the mammalian expression vector with the restriction enzymes NcoI/EcoRI and ligated into the prokaryotic expression vector phIS-parallel (Sheffield *et al.*, 1999) which was cut with the same enzymes. The prokaryotic expression vector was transformed into *E.coli* strain BL21(DE3). Protein expression was induced by adding 0.1 mM IPTG to a 400 ml culture of OD$_{600}$=0.6 for 16 hours at 20°C. The cells were washed with ST (20 mM Tris-HCl, 200 mM NaCl, pH 8.0), after which the cell pellet was subjected to a freeze-thaw process.
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cycle and was resuspended in 10 ml ice-cold ST containing 14 mg lysozyme, 1 mM PMSF, 0.1% (v/v) NP-40 and 25 U DNase (Benzoase Nuclease, Novagen). Protein was released by sonication and after centrifugation the soluble protein fraction was allowed to bind to His-Bind resin (Novagen) for 1 hour. The resin was washed at least two times with ice-cold ST. The fusion protein was eluted from His-Bind with ST containing 250 mM imidazole. The protein isolate was dialysed against a buffer containing 20 mM Tris-HCl and 100 mM NaCl, pH 8.0 after which the isolate was diluted to a final concentration of approximately 1 µM in 20 mM Tris buffer, pH 8.0. The sample was split in two fractions (A and B). To fraction A, 1 mM CaCl₂ was added to ensure saturation of the calcium sensor (Miyawaki et al., 1999). To fraction B, 1mM EGTA, pH 7.2, was added to scavenge any residual Ca²⁺. pH of the final solutions A and B were 8.0 and 7.9, respectively. From both fractions tiny droplets (<0.5 µl) were placed on a round cover slip (Ø 24 mm) by gently touching the cover slip with the tip of a syringe needle (25G: Ø 0.5 mm) containing the solution. Droplets were small enough so that the entire droplet would be visible in the field of view of a 10X enabling photobleaching of the droplet. The coverslip with the droplets was mounted in a culture chamber (Attofluar, Molecular Probes, USA), allowing access by the microscope objective. A lid was used to seal the chamber. To avoid evaporation of the sample during measurements, a larger drop of water (20 µl) was also put inside the chamber to saturate the chamber with water vapour. To check the functionality of the protein, two established methods to determine FRET were used; the spectra and the fluorescence lifetime of both fractions were measured. Acceptor photobleaching measurements according to the method introduced in this paper were performed four times on each fraction.

Preparation of HeLa cells
SYFP2 from the vector pSYFP2-C1 (equivalent to pEYFP-C1, Clontech, USA (Kremers et al., 2005)) was amplified by PCR to introduce a BamHI site downstream of the BglII site in the multiple cloning site. The NheI/BamHI fragment was inserted into the vector pECFP-N1 (Clontech, USA) cut by the same enzymes, thus creating a single open reading frame encoding a SYFP2-ECFP fusion protein with a 15 amino acid linker in between. Hela cells were seeded on coverslips and transfected the following day with 1 µg of this plasmid using Lipofectamine 2000 (Invitrogen, USA). The following day, cells were fixed with formaldehyde (4% w/v PFA in PBS) and washed with PBS. The coverslip was mounted on an object slide in mowiol (10% w/v polyvinylalcohol 4-88 and 25% v/v glycerol in 0.1 M Tris HCl (pH 8.5)) and sealed with nail polish.

Microscopy
Measurements were performed on an Axiovert 200M motorized microscope (Zeiss, Germany). The instrument is part of a multi-mode platform for fluorescence lifetime imaging microscopy (FLIM) and spectral imaging (SPIM), and is described extensively elsewhere (van Munster & Gadella, 2003; van Munster & Gadella, 2004). For intensity measurements, an AttoArc 100W mercury lamp (Zeiss, Germany) was used, the output intensity of which could be controlled remotely. The lamp was turned on at least half an
hour before measurements in order to avoid artefacts due to warming up of the lamp. Images were recorded on a cooled CCD camera (CoolSnap HQ, Roper Scientific, USA). 436/20 nm and 500/20 nm band-pass excitation filters, 455 nm and 525 nm long-pass dichroics, and 480/40 nm and 545/30 nm band-pass emission filters were used for CFP imaging and YFP imaging and photobleaching, respectively. Spectra were recorded using a 436/20 nm excitation and 460 nm long-pass emission filter (all filters from Chroma Corp., USA), using a slit-spectrograph (ImSpector, SpecIm, Finland), mounted in front of a CCD camera (ORCA II, Hamamatsu, Japan). Lifetime measurements were performed using the frequency-domain approach (Gadella et al., 1993) on the same instrument using a 442 nm Helium-Cadmium laser modulated at 75 MHz and the CFP filter set without excitation filter. Measurements on cameleon were performed using a 10X / NA 0.3 air objective (Zeiss, Germany). Measurements on the HeLa cells were performed with a 63X / NA 1.4 oil immersion objective (Zeiss, Germany).

Cross talk determination
To determine the spectral cross talk parameters for linear unmixing, purified solutions of the donor and acceptor fluorochrome were measured. Two slides were prepared with purified ECFP and EYFP protein solutions (Kremers et al., 2005) contained in round isolated vesicles (5 to 20 µm diameter), based on a protocol described elsewhere (Patterson et al., 1997). Basically, 50 µl of the protein solution was added to 450 µl octanol. The suspension was thoroughly mixed by repeatedly aspiring and releasing the mixture through a syringe needle (25G: Ø 0.5 mm). Immediately after mixing, 4 µl of the suspension was placed on an object slide and covered by a cover slip. To avoid sticking of the protein to the water-octanol interface, 5% (v/v) polyethylene glycol (PEG) 4000 was added to each protein solution before adding the octanol. Fluorescence intensities, corrected for dark-current and stray light, of both proteins were measured using both filter sets. After correction for differences in exposure time, it was found that the intensity of ECFP measured with the YFP filter set was 0.056% of the intensity measured with the CFP filter set. The intensity of EYFP measured with the CFP filter set was 4.5% of the intensity measured with the YFP filter set. This high percentage can be attributed to the extremely high intensity of the 436 nm line in the mercury spectrum compared to the low intensity around 500 nm. These parameters were used to construct a matrix, as described in (Zimmermann et al., 2003) for linear unmixing. To determine the cross talk of sensitised emission back into the donor channel, the value of c in equation 8 was determined by calculating the overlap integral using normalized ECFP and EYFP spectra recorded in a cuvette-based fluorospectrometer (Kremers et al., 2005) and spectral data provided by the manufacturer of dichroic and emission filters (Chroma, USA). A value for c of 0.0087 was found. For Qa, a typical quantum efficiency of EYFP of 0.65 was used (Tsien, 1998).

Performance of acceptor photobleaching
Before measurements, appropriate exposure times for donor and acceptor channels were established on another part of the slide then where the actual measurement was performed to limit unwanted photobleaching. Typically, exposure times were between 100 and 500
Gradual acceptor photobleaching

Cells were aligned and focussed using the CFP filter set and dimmed excitation light, avoiding any unnecessary exposure. A dark current recording with the excitation shutter closed was made preceding each measurement series to permit correction for dark current and stray-light. The actual recording sequence was performed with the excitation shutter open and the mercury lamp intensity at 100% and consisted of taking a recording with the CFP filter set immediately followed by taking a recording with the YFP filter set. After 60 seconds, during which the excitation shutter remained open to induce acceptor photobleaching, the entire cycle was repeated. After 20 cycles, the sequence was terminated and the excitation light switched off. Two stacks of 20 images each of both CFP and YFP channels resulted. The dark current recording was subtracted from all images in each stack. Measured intensities were corrected pixel-by-pixel at each time point using linear unmixing (Zimmermann et al., 2003).

Curve fitting
Fitting was performed in a user-selected region of interest (ROI) within the image, averaging the intensity within the ROI for each time point. Advantage of this approach as compared to analysis on a pixel-by-pixel base is that it is less sensitive to movement of objects during recording and to noise. Equation 2 was fitted to the measured acceptor intensity as function of time to obtain $I_{a,0}$, $I_{a,bg}$, and $\tau_a$. The $\tau_a$ value found was used in the fitting of equation 8 to the measured donor intensity as function of time to obtain $I_{d,0}$, $I_{d,bg}$, $\tau_d$, and $E_0$. Fitting was done by $\chi^2$ minimization using the downhill simplex method (Nelder & Mead, 1965; Press et al., 1994). The downhill simplex method used is not the most efficient method; here it was chosen for ease of implementation. Software for microscope control, image acquisition and fitting was written in C++ in MATLAB (Mathworks, USA) using the image processing library DIPlib (Quantitative Imaging Group, TU Delft, The Netherlands) for image display and ROI selection.

4.3 Results

Spectrum and lifetime of cameleon
To check the functionality of cameleon, spectra and lifetime of the YC2.1 construct were measured both with and without Ca$^{2+}$. Spectra in the presence and absence of Ca$^{2+}$ obtained with 436 nm excitation are depicted in figure 4.2. It can clearly be seen that the construct is functional as the spectrum shows a clear increase in YFP fluorescence when Ca$^{2+}$ is present indicating that FRET occurs. Lifetime measurements confirm this: phase lifetime decreased from 2.3 ± 0.2 ns in the absence of calcium to 1.9 ± 0.2 ns in the presence of 1 mM Ca$^{2+}$. Modulation lifetime decreased from 3.1 ± 0.3 ns to 2.9 ± 0.3 ns.

Acceptor bleach measurement of cameleon
To demonstrate the method introduced in this paper, it was applied to cameleon in the presence and absence of Ca$^{2+}$. On both fractions four measurements were performed. After curve fitting of equations 2 and 8 to the measured intensities in the presence of 1mM Ca$^{2+}$, values of $E_0$ of 0.35, 0.31, 0.28, and 0.31 were found. In the absence of calcium, values of $E_0$ of 0.15, 0.12, 0.18 and 0.15 were found. This results in average FRET efficiencies in the
presence and absence of calcium of 31 ± 3 % and 15 ± 2 %, respectively. The measured intensities as a function of time during acceptor photobleaching of one of the four measurements is shown in figure 4.3.

**CFP-YFP construct in HeLa cells**

To test the validity of the method on a biological sample, HeLa cells transfected with a CFP-YFP fusion protein were measured. An image of the HeLa cells is depicted in figure 4.4. The image shown is the first image using donor excitation and filter set of a series measured during acceptor photobleaching. Of this sequence of images, a region of interest was selected, depicted by the white square in the image. The average intensity inside this square is plotted as a function of time in figure 4.5. Curve fitting of equations 2 and 8 to the measured intensities gave a FRET efficiency $E_0$ of 0.28.

### 4.4 Discussion & Conclusion

**Evaluation of the method**

The FRET efficiencies found using the gradual acceptor photobleaching method introduced in this paper of cameleon in the presence and absence of calcium are 31 ± 3 %, and 15 ± 2 %, respectively. Although the spectra displayed in figure 4.2 do not permit an actual calculation of the FRET efficiency, the values found do not seem unrealistic, when looking at the spectra.
The phase and modulation lifetime of cameleon measured in the absence of calcium were $2.3 \pm 0.2$ ns and $3.1 \pm 0.3$ ns, respectively. In the absence of calcium, $1.9 \pm 0.2$ ns and $2.9 \pm 0.3$ ns were measured. Translating these lifetimes to FRET efficiencies is far from trivial. In the first place, the lifetime of CFP in the absence of FRET has to be well established. Values reported for CFP lifetimes vary from $2.4$ ns (Kremers et al., 2005), to $3.0$ ns (Rizzo et al., 2004). Secondly, lifetimes are not mono-exponential. This is true for both the lifetimes in the absence of FRET (Kremers et al., 2005; Rizzo et al., 2004) as well as for the measured phase and modulation lifetimes. Non-monoexponential decay can be attributed to the inherit non-monoexponential decay of the donor itself, as well as molecule-to-molecule variation in the actual FRET efficiency, for instance due to rotational freedom of the acceptor relative to the donor. In intensity-based methods, as method introduced in this paper, these molecule-to-molecule variations will still yield an accurate ensemble FRET efficiency since the individual contributions add up linearly. In lifetime measurements, however, given the highly non-linear nature of fluorescent decays, lifetimes of individual molecules do not simply add up. This makes it virtually impossible to reliably quantitate FRET efficiency from lifetime measurements. At this point we can only state that there is a clear reduction in phase and modulation lifetime in cameleon when compared to pure CFP and a further reduction by the presence of calcium, indicating that there is FRET occurring in cameleon in the absence of calcium and that the FRET efficiency increases by the addition of calcium.

The FRET efficiency of the CFP-YFP fusion in HeLa cells found using the method introduced in this paper was $28\%$. This would, according to equation 1 and a $R_0$ for CFP...
and YFP between 4.9 and 5.2 nm (Tsien, 1998) correspond to an intermolecular distance between CFP and YFP between 5.7 and 6.1 nm if orientation effects are not taken into account. Given the fact that both CFP and YFP are barrel shaped molecules with a diameter of 2 nm, and a length of 4 nm with the chromophore located in the centre (Rekas \textit{et al.}, 2002), and that a 15 amino acid linker is between them with a length, depending on the folding, of 2 to 4 nm, this value is what could be expected.

Advantages of the method are obvious when looking at the measurements and fitted curves in figures 4.3 and 4.5; had the existing method been used here, based on donor intensity before and after acceptor bleaching, there would have been a serious underestimation of the FRET efficiency. In addition, any artefacts in the behaviour of both donor and acceptor intensities as a result of acceptor bleaching would have gone unnoticed. Also, contrary to the existing FRET method based on direct acceptor photobleaching, the method is applicable in cases where complete acceptor photobleaching is not possible.

\textit{Measurement accuracy}

The accuracy of the FRET efficiency measured with the gradual acceptor photobleaching method is not easy to summarize in a confidence interval. The accuracy of the individual points through which the equations are fit, is mainly determined by photon statistics, i.e. Poisson noise. If exposure times are chosen in such way that the full dynamic range of the CCD camera is used, the noise is typically 1\% per pixel for a camera like the one used in this paper (QE = 60\%, full well capacity = 16000 e-, read noise = 8 e-, dark-current = 0.05 e- s\(^{-1}\), exposure times < 1 s). The noise in the average intensity over multiple pixels is the noise in one pixel divided by the square root of the number of pixels. In the example shown in figures 4.4 and 4.5, where the average intensity in a 16 x 16 pixel area is used, the noise would be reduced to 0.0625 \%. Needless to say that, purely based on these accuracies, the statistical variation in the parameter \(E_0\) found by the fitting will be extremely low as well. Although correct in a purely statistical sense, the analysis given above is not very useful. In practice, apart from variation in the sample itself, some errors due to deviations from the model are not unlikely. Looking at figure 4.3 it can be seen that both donor and acceptor intensity deviate from the fitted curve. The cause for these variations is unknown, but is most likely be the result of insufficiency of the model used. With the information available now, it is impossible to relate this to a measurement accuracy for \(E_0\) for this method. The variation of the \(E_0\) values found in the measurements of the cameleon construct of 10\% is an indication of what is realistic in practice. When using this method, instead of trusting a confidence interval derived purely on statistical grounds, it is important realize that there can be substantial variation in the sample and that measured curves can deviate from the model.

\textit{Mono-exponential bleaching}

Calculations in this paper were based on mono-exponential photobleaching. However, as can be seen in figure 4.3, bleach curves can deviate from a strict mono-exponential decay, even in relatively standard fluorescence microscopy applications. In principle, equations in this paper can be easily expanded to multi-exponential or stretched exponential models,
Gradual acceptor photobleaching

however, in general, little is known about photobleaching itself in relation to fluorescence microscopy. Photobleaching kinetics are dependent on, among other things, fluorochrome concentration, environmental circumstances of the fluorochrome, and wavelength, intensity and type (pulsed or continuous) of excitation (Song et al., 1995; Song et al., 1997). Even if the photochemistry of the fluorochrome and the processes leading to photobleaching are known, modelling of photobleaching beyond the approximation of a mono-exponential decay is extremely complicated due to the large number of unknown environmental factors encountered in practice.

One effect that may play a role in the photobleaching during FRET is that the fluorescence lifetime of the donor decreases with increasing FRET efficiency, which lowers the chance of excited state reactions that give rise to photobleaching. However, in a FRET pair, donor and acceptor should be regarded as one system. Whether excited state reactions will photobleach the donor, the acceptor, or both depends on the exact nature of the excited state reactions, which is something that is not well understood and is probably dependent on the environmental circumstances. Nevertheless, there is something known as a “protection effect” and a clear correlation between FRET efficiency and donor bleach rate has been shown. In fact, this is the basis for FRET methods based on donor photobleaching (Bastiaens & Jovin, 1998; Jovin & Arndt-Jovin, 1989; Jovin & Arndt-Jovin, 1989; Young et al., 1994). These effects have not been taken into account in the model. Therefore, it is important to realize, especially when dealing with large FRET efficiencies and high donor bleach rates, that the mono-exponential model used in this paper is a simplification that may give rise to deviations.

Dark states of YFP

General assumption in this paper is that a photobleached acceptor molecule loses its capacity to function as an acceptor. However, it should be kept in mind, especially when using fluorescent proteins such as YFP as acceptor, that they could display some unusual behaviour that may complicate FRET measurements. Fluorescent proteins have been found to transit between different states, including states with different excitation and/or emission maxima and dark states where they do not show any fluorescence at all (Blum et al., 2004; Dickson et al., 1997). The transfer to another state may occur spontaneously, or may be induced by irradiation at a particular wavelength. The degree in which fluorescent proteins suffer from these effects differs: fluorescent proteins have been described for which the effect is so strong that it is possible to effectively switch it on and off (Chudakov et al., 2004). For the acceptor used in this paper this effect did not play a role: When using excitation at 500 nm, the decrease in intensity of YFP has been found to be irreversible: after 15 minutes with no excitation light, the intensity did not come back, indicating that it is indeed photobleaching and not a transfer to a dark state. Had it not been photobleaching but a transfer to a dark state, the model used in this paper would only be valid if the acceptor would loose its absorption in this dark state. In this case the acceptor would loose its capacity to function as an acceptor just like it would when it were photobleached.
Quantitative use of FRET measurements

FRET is almost always measured on large ensembles of molecules. Unless it is a priori known that all donors exhibit a similar amount of FRET, such as in the examples in this paper, individual donors will not have identical FRET efficiencies; there will be a distribution of FRET efficiencies. In practice, the assumption can be made that there are two subpopulations: donors that are bound to an acceptor (FRET efficiency $E_0$), and donors that are unbound (FRET efficiency=0). The average FRET efficiency measured will be $\alpha E_0$, where $\alpha$ represents the ratio of the number of bound donors to the total number of donors. Care should be taken when relating measured FRET efficiencies to intermolecular distances using equation 1. For instance: a measured average FRET efficiency of 28% can mean that all donors exhibit 28% FRET, or that 28% of the donors exhibit 100% FRET. Without prior knowledge, the only conclusion that can be drawn is that the FRET efficiency measured is the minimum efficiency of the bound donor molecules. Even when 100% of the donors participate in FRET, relating a measured FRET efficiency to an intermolecular distance should be done with extreme caution: Because of distance distributions or rotational freedom, there may actually be a distribution of FRET efficiencies, the average of which can, because of the highly non-linear relation between the two, not simply be linked to an intermolecular distance. Methods based on relative intensities of donor and/or acceptor, such as the method introduced in this paper, all have in common that no distinction can be made between $\alpha$ and $E$. With techniques based on the decrease of fluorescence lifetime of the donor during FRET, however, it is possible to measure $\alpha$ and $E$ independently (Subramaniam et al., 2003; Verveer & Bastiaens, 2002).

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