Proposal of a new method to measure FRET quantitatively in living or fixed biomedical specimens on a laser microscope

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ABSTRACT

"Förster Resonance Energy Transfer", abbreviated "FRET", is a fluorescence phenomenon, which can be used to study and map co-localizations and dynamics of co-localizations at nanometer precision on a light microscope. FRET has been described as a "spectroscopic ruler". The efficiency of the radiationless energy transfer from an excited chromophore, the "donor", to another chromophore, the "acceptor", the excitation energy of which approximately matches the energy to be released by the donor, is dependent on the sixth power of the mutual distance between the two molecules in space.

We propose a new, non-destructive technique for measuring FRET quantitatively and at high spatial and temporal resolution on a laser scanning microscope:

Two laser beams of wavelengths suitable for the mutually exclusive excitation of the donor and the acceptor, the "donor beam" and the "acceptor beam", respectively, are intensity modulated by means of two electro optical modulators (EOM). The modulation patterns are rectangular at duty cycle ½. The modulation frequencies differ slightly. The acceptor beam is saturating the acceptor so that it cannot accept energy from the donor. The saturation is modulated in the same way as the acceptor beam. Since the donor beam also is modulated, though at a frequency slightly different from that of the acceptor beam, the intensity of the released donor fluorescence is modulated with the beat frequency of the frequencies of the two laser beam modulations and can be detected and interpreted in quantitative terms by means of a lock in amplifier.

Keywords: FRET, LSM, dynamic, non-destructive, Electro Optic Modulator, beat, saturation, modulation

1. INTRODUCTION

In the early 1920s, Franck and Cario observed and described that the energy stored in the electronic configuration of an excited chromophore, later named "donor", can be transferred without any molecular or atomic collisions and radiationlessly - i.e. without an interacting luminescence photon - to another chromophore, later named "acceptor", given that the amount of energy stored in the excited donor is suitable for the acceptor excitation [1].

This phenomenon, until the early 2000s commonly called (and wrongly so) "Fluorescence Resonance Energy Transfer", is today known as "Förster Resonance Energy Transfer", abbreviated "FRET".

FRET occurring, the luminescence of the acceptor instead of that of the donor luminescence is observed even if it is the donor - and not the acceptor - that originally had been stimulated by means of an external energy source.

Th. Förster interpreted the phenomenon quantitatively in quantum mechanical terms[2]. He concluded that, other than radiative energy transfer, i.e. the energy transfer via an interacting luminescence photon, FRET only can occur if the two chromophores are co-localized. The "FRET efficiency", i.e. the probability of the energy transfer by means of FRET, is dependent on the sixth power of the distance between the two molecules, i.e. $\left| \vec{r}^{\,6} \right|$.

During the 1960s, Förster's theory has been verified experimentally by Lubert Stryer and Richard P. Haugland [3].

The mutual distance of the FRET pair of chromophores, at which the FRET efficiency is 0.5, has later been named "Förster radius"; it is a characteristic value for any FRET pair of chromophores.

FRET has become a standard method in, e.g., fluorescence microscopy. In the life sciences, bioluminescent dye

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substances, which can be used as tracers in cells, feature a Förster radius between, roughly speaking, 1Å and 100Å. Thus, FRET indirectly extends the resolution of the light microscope into what normally is the domain of the electron microscope, and establishes a powerful tool for co-localization studies on live specimens. By means of FRET, it is, e.g., possible to observe live a variety of intracellular processes involving protein-protein interactions, i.e. functional mechanisms[4]. FRET has since the late 1990s even developed into a popular method for [Ca^{2+}] measurements[5-7].

If a measurement of the FRET efficiency in individual pixels or regions of interest (ROI) in digitized microscopic images can be interpreted quantitatively, then this interpretation is equivalent to a relative or, given an additional calibration measurement, an absolute distance measurement, at which the measured distances are ten to a hundred times smaller than the resolution limit of a conventional or even confocal light microscope.

However, the quantitative interpretation of microscopic FRET images is cumbersome.

Let, for example, be given the situation, where the distributions of donor and acceptor molecules in the specimen or on its surface are unknown. To properly determine the FRET efficiency in an ROI, at first, the donor fluorescence (DF) has to be measured in that ROI. Then, a second step, the acceptor destruction by intentional bleaching in the namely ROI, followed by a third step, the re-measuring the total DF, are required. Finally, the comparative analysis of pre acceptor bleaching DF and post acceptor bleaching DF may establish the basis for the quantitative interpretation. Besides the fact that this procedure may result in severe photo damage to any live specimen, it is not applicable in any situation, where the donor or the acceptor molecules move, e.g. by diffusion.

Alternatively, FLIM methods can be applied, where "FLIM" is an abbreviation for "Fluorescence Lifetime Imaging Measurement". Briefly, in FLIM-FRET measurements, the changes, which FRET induces to the mean decay time of the excited state of the donor, are registered. The time intervals to be detected by means of fluorescence spectroscopy are relatively short, at best some nanoseconds. Hence, these measurements are not an easy task and require special equipment, which, nevertheless, is available as industrial product.

Dynamic FRET measurements on living specimens, aiming at the detection of changing donor-acceptor configurations, can so far only be done either semi-quantitatively[8], at which the possible overlap of donor and acceptor fluorescence spectra will establish considerable problems, or by means of FLIM-methods[9].

The aim of this proposal is to present a new, alternative method for fully quantitative, dynamic, and non-destructive FRET measurements, e.g. on a laser microscope, a method not explicitly based on decay time registrations.

2. DESCRIPTION OF THE SYSTEM

2.1. Principle of operation

Based on a somewhat modified version of the "IMS-technique"[10-12], it may be possible to perform dynamic FRET-measurements in the following way (see Fig. 1):

The donor is being excited at a suitable wavelength λ_1, which must not at the same time excite the acceptor, and at the modulation frequency f_1, while the acceptor is being excited at λ_2, which wavelength must not at the same time excite the donor, and at the modulation frequency f_2. The modulation is performed by means of Electro Optic Modulators, "EOM"s. The intensity modulation patterns of the laser beams are rectangular. Since neither f_1 nor f_2 have to attain values that would have to be considered as large compared to what can be achieved by means of decent commercial EOM amplifiers, the rectangular shape of the intensity modulations of the laser beams will not be noticeably distorted at the edges. The only requirements are that the modulation frequencies are large compared to their difference frequency $\Delta f := |f_1 - f_2|$ and compared to the sampling frequency of the data acquisition, in case of imaging systems the pixel integration times. Obviously, Δf also is the frequency of the beat generated by the oscillations at f_1 and f_2.

The local oscillator signals generated by lock in amplifiers (LIA) "LIA 1" and "LIA 2" are applied in the usual IMS-way as modulation signals to the EOM amplifiers. However, they are also fed into the reference local oscillator input of a third LIA, "LIA 3", via a mixer stage. This third LIA is hence locked to the beat frequency Δf.

Two photomultiplier tubes (PMTs) detect the fluorescence. A dichroic mirror, designated as "dichroic emission filter" in Fig. 1, spectrally separates donor fluorescence and acceptor fluorescence. Quite a number of modern dye substances suitable as FRET couples feature narrow or even zero spectral bands of fluorescence overlap. However, even if it would not be possible in a given experiment to apply such dyes, the situation still can be optimized by means of suitable band pass filters mounted in front of the PMTs. For a graphic presentation compare Fig. 2.
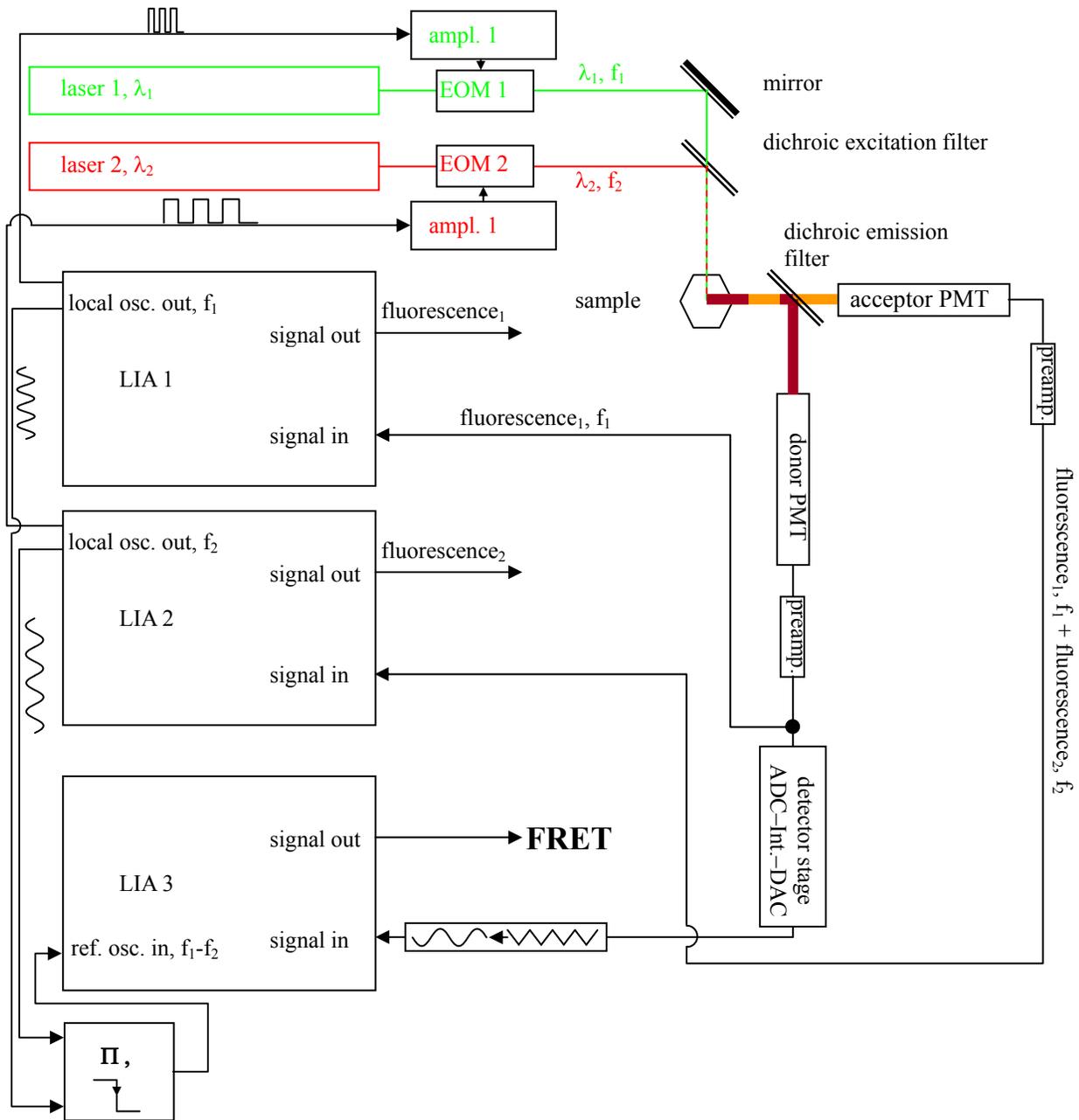
Fig. 1 The measurement principle (see previous page)

Two laser lines, the "donor light" and the "acceptor light", at wavelengths λ_1 and λ_2, which are suitable for the resp. excitation of the donor and acceptor molecules while at the same time not cross exciting the two chromophores, are modulated by means of two EOMs, "EOM1" and "EOM2". The modulation patterns, each generated by one of the outputs of the local oscillators of two lock in amplifiers, (LIA), "LIA 1" and "LIA 2", are rectangular. The frequency gap between the two modulations is small compared to the modulation frequencies f_1 and f_2. The modulation frequencies are large compared to the detection sampling times, in case of imaging systems the pixel integration times.

There is donor fluorescence if and only if donor and acceptor are *not* FRETing. Obviously, the donor fluorescence is modulated in a rectangular pattern at f_1.

Provided the flux of photons of the acceptor light is large enough, its modulation by "EOM 2" results in a rectangular shaped modulation of the ground state occupation of the acceptor molecules at frequency f_2. Any acceptor fluorescence generated by the "acceptor light" is, obviously, modulated at f_2.

Since f_1 and f_2 are slightly different from each other, there is another component of acceptor fluorescence, which is generated by FRET and which is modulated at f_1.

The signal from the acceptor PMT and its preamp are fed into "LIA 2".

As explained in later figures and the text, there is in addition a triangularly shaped modulation of the donor fluorescence at $\Delta f := |f_1 - f_2|$. This modulation has a triangular shape (see text and Fig-s.2-4).

The donor fluorescence is recorded by means of a photo multiplier tube, "PMT", the "donor PMT" and converted to a voltage signal by means of a pre-amplifier. This voltage signal is registered by means of "LIA 1". In addition, it is pre-processed in order to extract the triangularly shaped, slow modulation pattern, which then is transformed into a sinusoidal pattern before being registered by a third LIA, "LIA 3", which is tuned by means of the "local oscillator out" signals of "LIA1" and "LIA2" as well as a multiplier and a low pass filter to detect signals at Δf. As explained in the text and the other figures, the output signal provided by "LIA 3" allows to quantitatively determine the FRET efficiency.

While the outputs from "LIA 1" and "LIA 2" are not necessary for the determination of the FRET efficiency, they can, obviously, nevertheless be useful.

2.2. A suitable test preparation

A suitable test preparation is, in the simple most case, a solution of a substance that consists of bound FRET-donors and FRET acceptors in a cuvette. Dependent on which FRET conditions are to be simulated, a certain number of spacers [3] is separating the donor and the acceptor and keeping them at a constant mean distance[§]. A suitable test preparation for studying the phenomena on a laser scanning microscope are small containers of solutions with different FRET conditions as described in Fig. 5.

[§] Large chromophores, due to their complicated 3D-structure, can perform rotations in space and thus periodically change the distance between donor and acceptor on time scales negligibly short for the further considerations.

I_{Laser}, arbitrary units and suitable relative magnitude
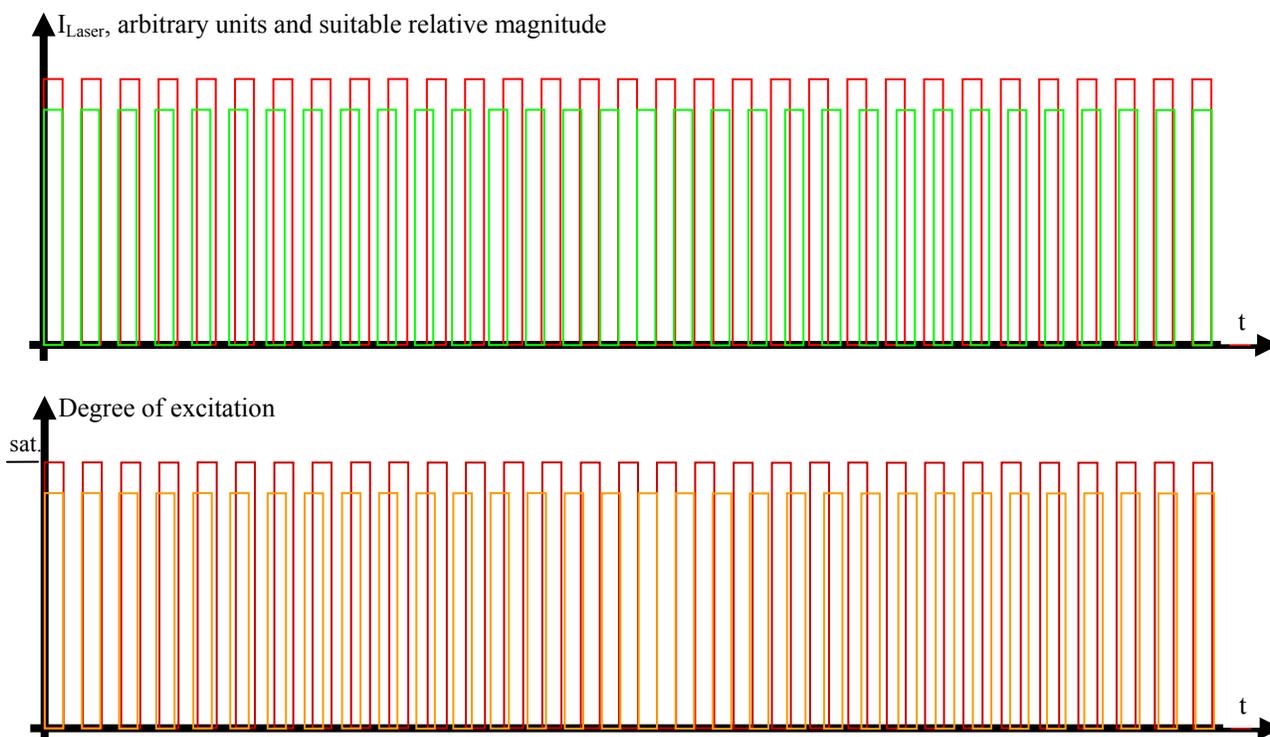
t

Degree of excitation

sat.

t

Fig. 2 The laser beam modulations and degrees of excitation
In the upper part of the figure, the rectangular shaped modulation pattern of the laser beam is shown. The frequency of the modulation of the laser light exciting the acceptor chromophores, marked in red, is slightly different from the frequency, at which the laser beam exciting the donor chromophores is modulated.
The respective degrees of excitation of the ensemble of the acceptor molecules – in deep red colour – and donor molecules – orange – are shown in the lower part of the figure. The time delays between the stimulating laser pulses and the resulting degrees of excitation are negligibly small compared to the times matching the modulation frequencies.
As will be shown later, it is *not* absolutely necessary that the laser beam exciting the acceptor molecules would be strong enough to attain a degree of excitation of the ensemble of the involved acceptor molecules close to saturation.

2.3. The molecular processes
2.3.1. Case A, "ideal FRET conditions", donor and acceptor close enough to each other for optimal FRET

Donor and acceptor are FRETing whenever possible, i.e. when the acceptor is not in its excited state, i.e. has not been excited by the laser beam at λ_2 right ahead of the moment, when the donor can transfer the energy to the acceptor. Since both modulation frequencies are "nearly identical", the phase difference between both rectangular modulations is going to oscillate at the beat frequency. Thus, also the FRETing oscillates at the beat frequency. The amplitude of this oscillation is going to be quite strong if the power of the laser beam at λ_2 is sufficiently large, hence the degree of excitation of the involved acceptor molecules close to saturation.
Studying the situation in detail, one has to consider three cases (see Fig 2, Fig. 3 and Fig. 4)
a)
Donor excitation and acceptor excitations are in phase.
In this case, the donor fluorescence as well as the acceptor fluorescence are being modulated by f_1 or f_2, at which the modulation will be quite deep. There are strong signals in LIA1 and in LIA2 before the respective amplifier stage[**].

[**] Note! In this simplified analysis it is assumed that during a) and the parts of c), when the direct excitation of the acceptor by the laser beam at λ_2 and FRET excitation of the acceptor are competing, laser excitation is dominantly strongly. A quantitative analysis is provided later on in this article.

b)

Donor excitation and acceptor excitation are entirely counter phase.

Then, the acceptor is fluorescing all the time, since it is excited all the time, either by FRET or by the laser beam at λ_2. Donor fluorescence is close to zero all the time, since the donor excitation energy is transferred to the acceptor. The modulation depths of both, donor and acceptor fluorescence are going to be small. Neither in LIA1 nor in LIA2 will there be a considerable signal before the respective amplifier stages.

c)

All intermediate states.

a) and b) are going to be present, each during its resp. part of the oscillation period.

In other words:

The average values of the donor and acceptor fluorescence intensities - integrated over many modulation periods - are going to oscillate at the beat frequency. The envelope of this beat oscillation is a triangular signal, which LIA3 registers if the signal by the donor fluorescence PMT has been processed by a detector stage / "AD-Integrator-DA" circuit before de-modulation.

2.3.2. Case B, "ideal non-FRET conditions", donor and acceptor far enough from each other to render any FRET process impossible

Here, LIA3 is not going to detect anything, the LIA3 output is zero. Please, note that the signals of both PMTs are NOT directly mixed! Even if the total fluorescence still shows a strong beat signal, generated by the two directly excited fluorescence patterns, exclusively, "dichroic mirror 2" separates both oscillations, f_1 and f_2

2.3.3. Case C, any intermediate situation

The output signal of LIA3 is going to be of intermediate strength.

2.4. Qualitative analysis of FRET measurements

The following description is, again, based on the assumption that the modulation frequencies are so small that their periods are long compared to the optical relaxation times of the chromophores. Moreover, the difference between the two frequencies is assumed to be small compared to the frequencies themselves. At the same time, both, the periods of the individual modulation frequencies and the period of the difference frequency should be sufficiently short compared to the pixel sampling times. 15 MHz and 12 MHz as f_1 and f_2, hence 3 MHz as $\Delta f = |f_1 - f_2|$ are suitable even for pixel sampling times in the microsecond region.

In a genuine sample for laser scanning microscopy, the different cases (A, B and C, see above) are usually going to be realized locally in the preparation (normally pixel wise, for reasons of clarifications as ROIs in Fig. 5). Dependent on whether there is any FRET or not the pixel values generated by LIA3 detection are smaller or larger. When performing a scan, the signals from the three LIAs will produce three image frames. Channel 1 and 2 are directly acquired. The analog signals by the donor-PMT and LIA1 as well as by the acceptor-PMT and LIA2 [††] are registered via the ADC of the CLSM.

Channel 3, however, exclusively represents the degree of FRET. In other words, the more efficient FRET is in the resp. pixel location, the larger the respective pixel value is going to be.

The entire measurement process can be developed in three stages, I, II and III, see Fig. 5:

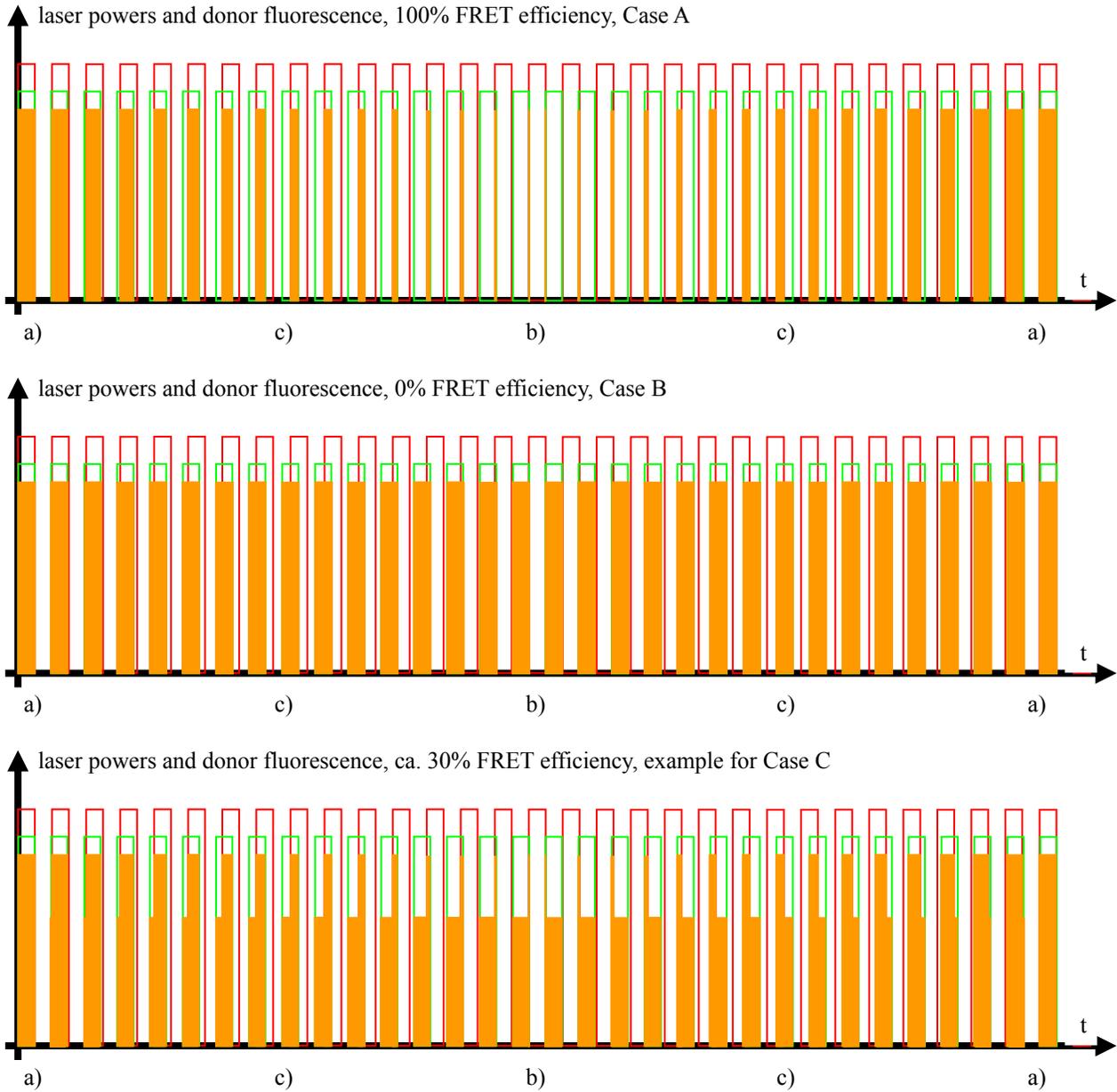[††] respectively the averages of their beat values.

Fig. 3   Donor fluorescence as a function of the FRET efficiency
The FRET efficiency is detected via the donor fluorescence. In this figure, the donor fluorescence, symbolized by the orange rectangles, is shown as a function of the time and the FRET efficiency. Obviously, un-altered donor fluorescence is generated only when there is donor excitation *and* **not** any FRET is happening at the same time. Depending on the FRET efficiency, a certain fraction of the excitation energy stored in the excited donor molecules is not emitted as fluorescence but radiationlessly transferred to the acceptor. This is the reason for the shapes of the geometrically distorted orange rectangles (see text for detailed explanation).
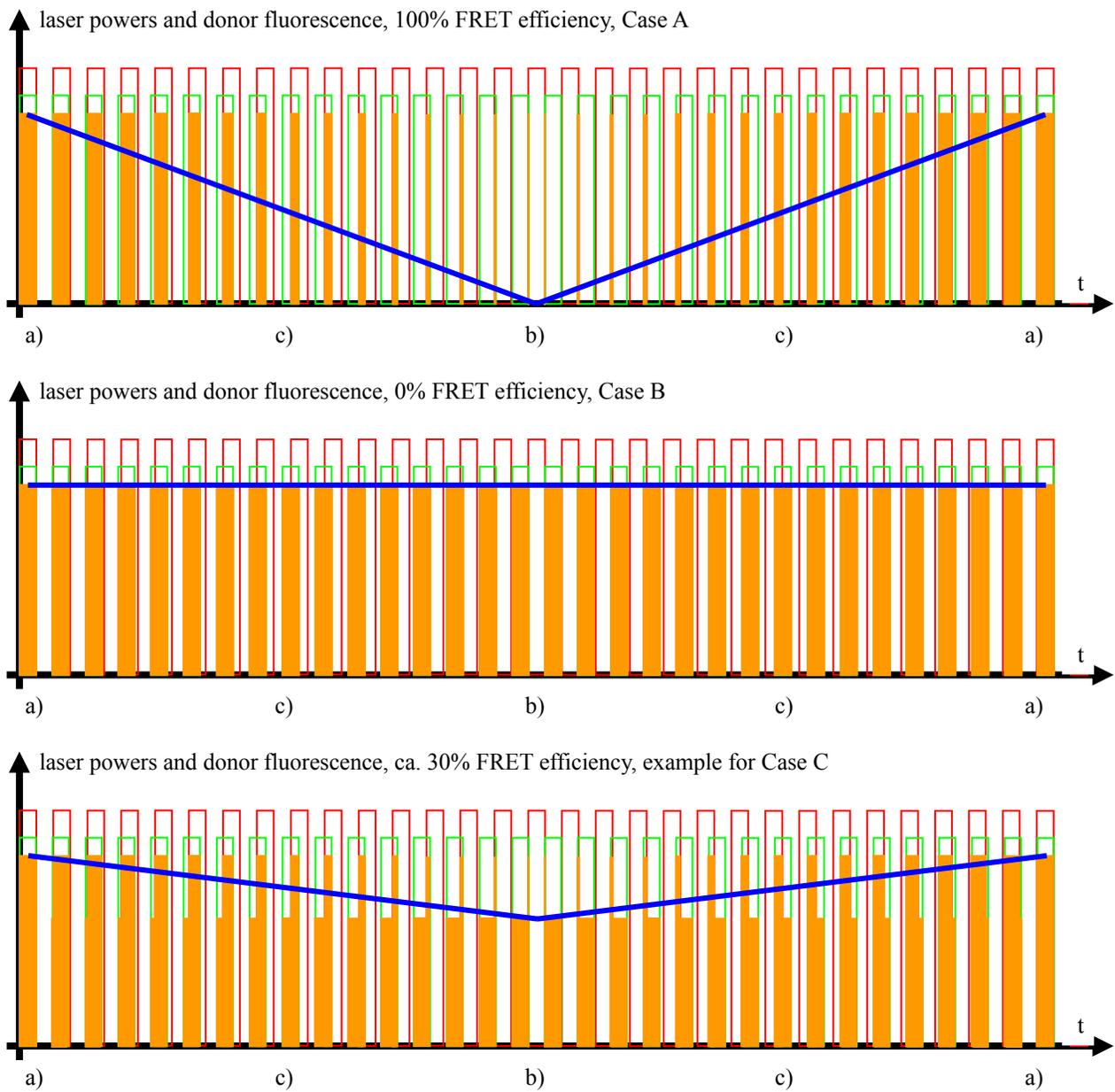
Fig. 4   The FRET signal
The blue curves show, in three different cases, the donor fluorescence as measured by the donor PMT and filtered by the detector stage. The blue curves contain all the information necessary for the quantitative FRET analysis.
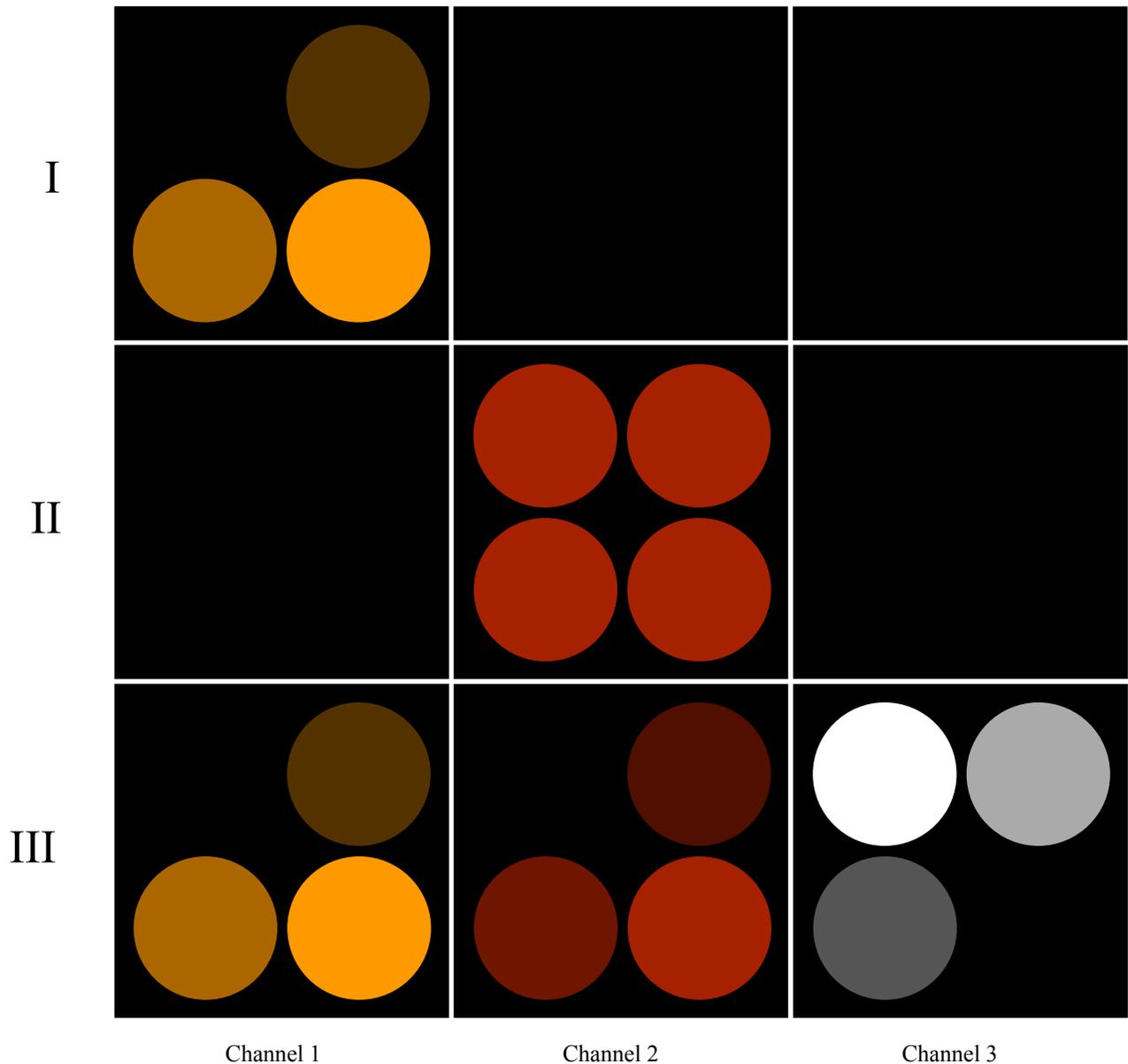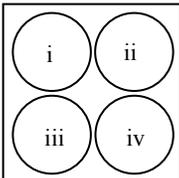
| | Channel 1 | Channel 2 | Channel 3 |

Fig. 5   FRET on a microscopic test preparation
Donor fluorescence orange shaded, acceptor fluorescence deep red shaded, FRET grey shaded.

Read:

i	ii
iii	iv

i: optimal FRET (Case A), ii: somewhat less FRET (example for Case C), iii: even less FRET (another example for Case C), iv: no FRET at all (Case B)l

In this figure, the image of a hypothetical test preparation is shown when scanned as quadratic frame and illuminated with different excitation wavelengths. Four circular regions in the scanned field contain uniform specimens with different FRET conditions. Rows from I to III show the result of illuminations with respectively λ_1, only, λ_2, only, and finally simultaneously with λ_1 and λ_2, at which the respective laser beams are modulated as outlined in the text. Channel numbers are related to the LIA numbers as defined in Fig. 1 and the text.

I

At first, the preparation is illuminated by (λ_1, f_1), only, and the phase on LIA 1 is optimized for maximum intensity in channel 1 (that part of the donor fluorescence that is not "FRETed away"). Dependent on the FRET degree, the signal will be more or less intensive, see i, ii, iii, iv in Channel 1. The FRET fluorescence of the acceptor cannot be seen in Channel 2 since it does not oscillate at f_2 but at f_1, instead. The signal is zero in Channel 3.

II

The same is being done at (λ_2, f_2) (LIA 2 is being optimized for directly excited, i.e. non-FRET acceptor fluorescence). All the regions (i, ii, iii and iv) are going to shine equally strong since the acceptor is being excited directly and with the correct modulation frequency f_2. Channels 1 and 3 are entirely dark.

III

Finally, the preparation is illuminated simultaneously by (λ_1, f_1) and (λ_2, f_2). In region i, the donor fluorescence in Channel 1 is zero. The reason is FRETing to the acceptor. The value of Channel 2 in i is zero since the modulation depth of the acceptor is zero[‡‡]. In Channel 3 i, the signal is strong since the beat amplitude is large (remember, that the beat frequency is so large that its period is short compared to the pixel dwelling time). In ii, iii, and iv the FRET efficiency is decreasing more and more.

By means of this method, the FRET efficiency can be measured quantitatively and independent of the optical decay times of the chromophores. Of course, the shorter the acceptors decay time is, the larger the intensity of the laser beam at (λ_2, f_2) has to be in order to attain saturation of the excited state in the acceptor molecules. Pixel value 255 (respectively. 4095 in a 12 bits instrument) indicates 100% FRET efficiency; pixel value zero indicates 0% FRET-efficiency. The *quantitative* approach in terms of formulas is presented in a later paragraph.

2.5. Principal problems

Obviously, it is sometimes undesirable to operate the laser beam at (λ_2, f_2) at high intensities to attain perfect saturation of the excited state of the acceptor chromophores. However, *saturation is not necessary* for the quantitative analysis as will be shown en détail in a later paragraph. In qualitative terms: It is possible to circumvent acceptor fluorescence saturation by registering a sequence of images at increasing but not saturating laser power and to extrapolate a fit curve to saturation intensities during post experimental data analysis.

For the method to be functional, a large extinction coefficient of the EOMs is necessary. At frequencies beyond 20MHz, which is necessary for following *rapid* FRET processes on a millisecond timescale demanding rapid scanning, this, unfortunately, translates into several ten thousands of US-Dollars per amplifier.

2.5. A suitable test preparations

It is technically possible to synthesize both, donor-acceptor-molecules with ideal FRET-conditions as well as donor-spacer-acceptor groups with spacers of well defined lengths to simulate different degrees of the FRET efficiency. The dye substances "Cy3" or "Cy3.5" and "Cy5" have close to ideal excitation and emission spectra as FRET couple for test experiments. The Förster radius of the Cy3.5-Cy5-couple is assumed to be nearly identical to the Förster-radius of the Cy3-Cy5-couple. "Spacers", which can be grouped between donor and acceptor, are going to be approximately 35Å in length, which is a suitable value. The synthesis of a similar substance has been described [3].

2.6. Principal problems and the quantitative analysis of FRET measurements

In this paragraph, some principal problems are being outlined, which are direct consequences of the nature of the FRET phenomenon or the properties of the preparation. Unavoidably, these problems are obstacles *independently of the method*, which is used in order to measure FRET. The analysis of these problems leads in a quite natural way to the quantitative analysis of the measurement procedure.

2.6.1. A problem *independent of the measurement technique*: Clustering

One problem is possible clustering of either donor or acceptor or both, donor and acceptor molecules.

Example: *Two* acceptor molecules are located in the 50% FRET vicinity from *one* donor. This situation *perfectly* simulates the *100%* co-localization of *one* FRET donor and *one* FRET acceptor. The *apparent* value for the FRET efficiency then has to be re-normalized. If there are exclusively acceptor clusters, this re-normalization is not too problematic. Using direct fluorescence intensity measurements via LIA 2, it is possible to find out how much the acceptor fluorescence intensities in one pixel differ from the respective values on another pixel. This measured factor then is the re-normalization factor. However, it might also happen that *many* acceptor molecules are located close to *one*

[‡‡] NB! In situation III, where both laser beams are simultaneously active, regions i in Channel 1 and 2 will *in praxi* not be *perfectly* zero. Firstly, FRET efficiency never attains really 100% so that there will be a small fraction of rest donor fluorescence at f_1. Secondly, depending on the strength of the laser beam at (λ_2, f_2), there will unavoidably be a rest modulation depth of acceptor fluorescence at f_2.

donor, but not all at really the same distance but at different lengths so that there are going to be a different FRET efficiency for each of these acceptors. Then, the situation is very difficult and no solution can be suggested at present. Of course, it is possible to calculate a "global" FRET efficiency even then. However, any interpretation of this value will be quite un-safe.

The situation turns out to be more complicated if there are *donor* clusters. In this case the least complicated situation is given by *several* donors located close to *one* acceptor. Even in this least complicated scenario, the calculation of the mutual distances between the donors and the acceptors is not possible. Neither is it possible to perform any re-normalization.

The most complicated case, in which there are *simultaneous* arrangements of donor *and* acceptor clusters, at present establishes an un-solvable problem for any *quantitative* analysis[§§].

2.6.2. *Method dependent* FRET problems - a fully quantitative system

A somewhat subtle problem appears, even in the simple most situation of a specimen entirely free from any clusters – and all the further considerations in this paragraph are restricted to this situation –, when performing a closer analysis of the suggested measurement technique:

The method is entirely based on the beat of the donor fluorescence. This beat is generated by exposing the acceptor molecules to an f_2-modulated laser beam at the proper excitation wavelength λ_2. By means of this laser beam the capacity of the acceptor molecules to participate in FRET is being modulated.

The *minimum value* of the beat in the donor fluorescence is generated when there is no direct excitation of the acceptor molecules while the donor excitation is at its maximum[***]. Consequently, the *minimum value* of the beat in the donor fluorescence is exclusively dependent on the donor excitation and the mutual distance between the donor and acceptor molecules. Thus, this measurement value is trustworthy.

Some doubts could possibly be expressed concerning the trustworthiness of the *maximum value* of the beat in the donor fluorescence. This value can exclusively be measured while the direct acceptor excitation is so dominant that all the acceptor molecules are in the excited state when the donor molecules are ready to start a FRET process. In other words, the direct excitation has to dominate the FRET excitation. For this purpose, Laser 2 has to drive the excitation of the acceptor molecules into saturation. Even if this technically is possible and even if modern dye substances are relatively resistant vs. bleaching, a biological preparation possibly would suffer from this treatment. Moreover, from a purely *esthetical* point of view, this approach appears to be somewhat *brute force*.

However, there is a solution to this problem:

Firstly, the situation can be optimized by performing two pre-experimental measurements aiming at the proper adjustment of the powers of the two laser beams, which have to be settled individually so that the excitations of the donor or acceptor molecules are just attaining saturation[†††]. Thus, the amplitude of the beat to be measured later on is maximized[‡‡‡].

Secondly, the idea now is that the amplitude of the beat of the donor fluorescence always is a quantitative *relative* FRET scale, even if the two extremes in the donor fluorescence beat, i.e. the amplitude of that oscillation, which is going to be fed into LIA3, *do resp. does not express* the difference between "*complete FRET*" and "*zero FRET*" but the difference between "*practically strongest possible FRET*" and "*rest FRET*". This will be proved mathematically:

Let *F* be the FRET efficiency. Then

[§§]However, even then it is still possible to *qualitatively* decide whether there is any FRET or not.

[***]Again, it is assumed that the optical relaxation times of the chromophores are short compared to periods of the oscillations at f_1 or f_2.

[†††]This setting of the powers is attained by observing the increase of the individual fluorescence signals. Exceeding the value for excitation saturation results in a reduction of the signal passing the LIAs, since the excitation modulation depth attainable by EOM modulation is going to decrease.

[‡‡‡]In order to do this measurement, it is advantageous to provide some region on the cover slip or in the Petri dish close to the preparation, where a small quantity of test substance with known FRET efficiencies NULL and ONE are mounted. In most cases, to do so will not establish any practical problem.

$$0 \leq F = 1 - \frac{M}{N} \leq 1,$$

where N is the hypothetical maximum of the donor fluorescence (no acceptor molecule is accepting FRET energy) and M is the donor fluorescence when FRET is occurring.

What is happening when there is induced a direct acceptor excitation by means of Laser 2, in mathematical terms, is a reduction of the FRET efficiency. If the power of Laser 2, $J(\lambda_2)$, is increasing, M *is increasing, too*. In the limiting case of an infinitely large $J(\lambda_2)$, M is approaching N,

$$\lim_{J(\lambda_2) \to \infty} M(J(\lambda_2)) = N.$$

The problem, now, is to establish a functional relationship between M, N and $J(\lambda_2)$. Considering the conditions for $J(\lambda_2)$ at zero or at maximum the following formula is suggested:

Define $M_0 := M(J(\lambda_2)=0)$, then

$$M(J(\lambda_2)) = M_0 + e^{-\frac{1}{J(\lambda_2)}}(N - M_0).$$

The FRET efficiency as a function of the power of the laser exciting the acceptor molecules then is

$$F = 1 - \frac{M_0 + e^{-\frac{1}{J(\lambda_2)}}(N - M_0)}{N}.$$

In the limiting case of $J(\lambda_2)=0$ this equation is identical to the definition of the FRET-efficiency (see above), while the FRET-efficiency converges versus zero, when $J(\lambda_2)$ is increasing to large values.

The actual measurement value, in other words the value as delivered by LIA3, is the amplitude of an envelope of a beat signal

$$A = k(M(J(\lambda_2)) - M_0).$$

where k is a proportionality factor.

Assuming two places in a preparation with different FRET efficiencies F_1 and F_2, the ratio of these efficiencies is

$$\frac{F_1}{F_2} = \frac{N - M_{0_1}}{N - M_{0_2}};$$

where (see above) it is assumed that N_1 and N_2 are identical and are named N.

The question now is, what the ratio of the LIA3 signals at the two places look like. Inserting the respective terms in the definition equations renders:

$$\frac{A_1}{A_2} = \frac{k\left(e^{-\frac{1}{J(\lambda_2)}}(N - M_{0_1}) + M_{0_1} - M_{0_1}\right)}{k\left(e^{-\frac{1}{J(\lambda_2)}}(N - M_{0_2}) + M_{0_2} - M_{0_2}\right)} = \frac{N - M_{0_1}}{N - M_{0_2}}.$$

In other words

$$\frac{F_1}{F_2} = \frac{A_1}{A_2}.$$

Thus, the relative FRET efficiencies at different locations in the preparation are directly related to the amplitudes, i.e. the pixel intensities, in the LIA3 image at these places.

If one now has a location in the preparation with a microscopic drop of a solution of test substance, where the *absolute* FRET efficiency is known, the absolute FRET efficiency at all other locations in the same preparation can be determined by the relative quantification shown above. The same can, of course, be done by means of separate test preparations, where the FRET efficiency is known. The only demand is that the laser effect be unchanged between the experiment and the control measurements.

The system, hence, allows for fully quantitative and dynamic measurements.

Here, it should be mentioned that the quantitative analysis of FRET images opens for quantitative measurements of the dynamics of intracellular $[Ca^{2+}]$ at confocal spatial resolution since FRET based $[Ca^{2+}]$ indicators are available by now [5, 6]. An old and severe problem in the study of intracellular processes will thus be brought considerably closer to its solution.

2.7 FRET and Multi Photon Laser Scanning Microscopy

Common commercial tunable titanium sapphire lasers have pulse rates of approximately 80 MHz.

Using one or two phase modulating EOMs, a beat situation can be simulated. The phase modulation would accelerate or retardate one or both of the laser beams. Thus, a beat can be simulated by electronic means, provided two titanium sapphire lasers are available and if the two chromophores in the respective donor acceptor couple can be excited *selectively*, each chromophore by one of the beams. The method then could e.g. be used for semi quantitative or even quantitative $[Ca^{2+}]$ measurements employing the "Cameleon" dye, at which CFP and YFP can be excited selectively by specific Ti-Sap laser wavelengths[5, 6].

3. RESULT

We have presented the outline of a new, non-destructive method for the quantitative and fast measurement of FRET processes. The method is independent of any specific method of observation and may be applied to imaging as well as purely photometric methods. The method promises to be a useful tool, noticeably in high resolution light microscopy.

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