Fluorescence resonance energy transfer analysis of protein–protein interactions in single living cells by multifocal multiphoton microscopy

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Abstract

Fluorescence resonance energy transfer (FRET) resolved by multifocal multiphoton microscopy (MMM) was successfully used to measure transport phenomena in living cells. We expressed different pairs of CFP-/YFP-fusion proteins involved in retrograde Golgi-to-ER transport to analyze sorting of the occupied KDEL-receptor into retrograde transport vesicles triggered by application of the external cholera toxin mutant CTXK63. FRET observed as a sensitized emission of the acceptor was confirmed by acceptor photobleaching and the dequenching of the donor was measured. FRET--MMM data obtained from single cells were compared with bulk cell experiments employing spectrofluorimetry. The importance of controlling the degree of overexpression of CFP-/YFP-fusion proteins for FRET analysis is stressed in this article. Using MMM we showed for the first time that FRET can be measured across the Golgi membrane. Finally, FRET--MMM records performed continuously over 2 h allowed to analyze intracellular retrograde transport and sorting events and to discuss these mechanisms on a single cell level.

Keywords: Fluorescence resonance energy transfer; Multifocal multiphoton microscopy; CFP; YFP; KDEL-receptor; Cholera toxin; Protein–protein interactions

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1. FRET–MMM analysis of protein–protein interactions in living cells: experimental considerations

The ‘green revolution’ initiated by the introduction of the green fluorescent protein (GFP) from Aequorea victoria (Chalfie et al., 1994; Cubitt et al., 1995; Tsien, 1998) and the later developments of GFP-mutants possessing different spectral properties (Pollok and Heim, 1999) offered the possibility of simultaneous expression of donor and acceptor fusion proteins in the same cell and allowed measurement of their interactions by fluorescence resonance energy transfer (FRET) (Miyawaki et al., 1997; Mitseli and Spector, 1997; Siegel et al., 2000). At present, the combination of CFP (donor) and YFP (acceptor) fusion proteins are particularly useful. This effective FRET pair can be used to monitor the proximity of the two attached fluorescent proteins in 3–6 nm (Tsien, 1998). Co-expression of CFP- and YFP-fusion proteins has been successfully used to analyze short-time changes in protein–protein interactions, e.g. oligomerization, colocalization, complex formation (Gadella and Jovin, 1995; Blackman et al., 1995; De Angelis et al., 1998, Ellenberg et al., 1999), activation of protein kinases (Ng et al., 1999) and mapping of enzyme activities in living cells (Bastiaens and Pepperkok, 2000).

FRET was first described by Förster (1948). It has become extremely important for modern cell biology, because FRET allows to measure distances between molecules on a scale of a few nanometers. This is far below the resolution limit of modern optical far field microscopy, which currently is at approximately 100 nm (Hell et al., 1997; Nagorni and Hell, 1998; Klar et al., 2000). Förster’s theory explains FRET as a dipole–dipole interaction between neighboring molecules and derives the dependence of the energy transfer efficiency \( E \) on their actual proximity \( R \). For efficient FRET the distance \( R \) between these two molecules, the excited donor \( D \) and the fluorescent acceptor \( A \), is typically 2–7 nm. The direct non-linear dependence between \( E \) and \( R \) can be described as

\[
E = \frac{1}{1 + (R/R_0)^6}
\]

\( R_0 \) is by definition the distance between the \( D \) and \( A \) for \( E = 0.5 \). \( R_0 \) reflects the properties of a particular \( D/A \) pair including acceptor quantum yield, spectral overlap between \( D \)-emission and \( A \)-excitation and the relative spatial \( D-A \) orientation.

\( E \) can be obtained directly from the experiment:

\[
E = 1 - \frac{I_{DA}}{I_D}
\]

with \( I_{DA} \) the fluorescence intensity of the donor in the presence of the acceptor and \( I_D \) the fluorescence intensity of the donor alone. The extreme sensitivity of the FRET process on the distance between molecules (6th power dependence on \( R \)) renders it a very useful tool for the resolution of intracellular arrangement and dynamics of biological molecules. However, fluorochrome bleaching and induction of cell damage limit the application of the FRET technique for the study of long-term (minutes to hours) processes of transport or signal transduction in living cells. We describe here, that these problems can be ameliorated by:

- applying two-photon excitation instead of one-photon excitation (Denk and Svoboda, 1997); and
- introducing multifocality to multiphoton microscopy (Bewersdorf et al., 1998; Straub and Hell, 1998a,b; Straub et al., 2000).

In the following we analyze some examples and show that the combination of the two methods in FRET–multifocal multiphoton microscopy (MMM) presents significant progress on the way to study protein–protein interactions in living cells over a prolonged period of time. MMM not only reduces the radiation damage of the cells in long-term experiments, but also increases the resolution in the area of interest (for details see Straub et al., 2000). In contrast to single photon excitation, the two photon excitation confines imaging to a very thin (~1 μm) plane and favors the analyses of cellular compartments (e.g. the Golgi-region) along the z-axis, diminishing the
background out of focus excitation and making it convenient for the reconstruction of three-dimensional images. The donor (CFP) was two-photon excited at 800 nm infrared light, and the CFP-emission at $\approx 470$ nm or, in case of FRET, the acceptor (YFP-) emission at $\approx 525$ nm could be separated easily using suitable emission filters. The physics of two-photon excited FRET between CFP and YFP as well as for the experimental set-up are presented in Fig. 1, the latter being discussed in detail in the next section.

Experimentally FRET can be measured by:

1. sensitized emission of the acceptor upon donor excitation;
2. the decrease in D fluorescence intensity or life time induced by the presence of the acceptor; and
3. FRET can be confirmed by dequenching donor fluorescence as an increase in donor fluorescence or donor life time upon acceptor photobleaching.

Different laboratories utilize different experimental set-ups to measure FRET (described in Wouters et al., 2001).

In this review we will discuss sensitized acceptor emission as well as acceptor (YFP) photobleaching experiments in single living cells during toxin induced retrograde transport. In particular, we consider the question of whether FRET between a given pair of CFP- and YFP-fusion proteins gives sufficient information to draw conclusions as to the functional relevance of such interactions. For this reason we have analyzed changes in FRET-signals for different pairs of CFP-/YFP-fusion proteins under steady-state conditions and after a short pulse of cholera toxin. In the examples given, we confirm by FRET-MMM analyses that the retrograde vesicular transport from the Golgi to the endoplasmic reticulum (ER) includes the rearrangements of protein–protein interactions on the Golgi membrane induced by the KDEL-receptor interaction with a specific ligand. Cholera toxin, which is rapidly taken up by the cells and whose A subunit possesses a C-terminal KDEL-signal, binds to the KDEL-receptor (KDELr) in the Golgi (Majoul et al., 1996, 1998). CFP- and YFP-fusion proteins used for FRET measurements and their intracellular localization are depicted in Fig. 2. A mutant cholera toxin unable to increase cellular 3’,5’-cAMP was used in these experiments (Fontana et al., 1995).

Drawing conclusions with respect to the addressed cellular function on the basis of FRET data between pairs of CFP-/YFP-fusion proteins, as well as performing comparisons with normal cellular functions in living cells is justified only if the fusion proteins behave similar to the corresponding endogenous wild-type proteins. Therefore, the experimental controls listed below need to be performed:

1. the expressed fusion proteins used in the experiments should reveal the same subcellular distribution as the WT-proteins;
2. the expression of the fusion proteins per se should not induce or inhibit testable cellular functions (e.g. kinetic of cholera toxin Golgi to ER transport in this particular work); and
3. unspecific CFP- and YFP-fusion proteins expressed in the cell should not create significant background FRET-signal (due to overexpression, pH shift, etc.).

Usually, one wishes to express the pairs of fusion proteins in a large number of cells. Depending on the cell type the Ca-phosphate method as well as transfection reactions based on cationic lipids (e.g. lipofectin, lipofectamin, Gen Porter, etc.) can be used. In some cases the microinjection of cDNA in the nucleus or mRNA in the cytoplasm may be of advantage. In our hands, the most effective transfection method was electroporation which results in transfection rates of 80% and higher (for details see Majoul et al., 2001). Soon after transfection (occasionally already at 6 h) the overexpression of one or more CFP-/YFP-fusion proteins may severely impair cellular function. In theory, inducible promoters can be used to regulate the degree of expression. Tetracycline-regulated promoters cannot be used in combination with two-photon excitation of CFP, as even traces of tetracycline create a strong
fluorescent background. Expression systems induced by ecdysone or hygromycin may be more suitable. In our hands, the selection of an appropriate time window after transfection gave the best results. We expressed the fusion proteins under control of the relatively strong CMV-promoter. Conventional fluorescence microscopy was used to control the time-course of expression and to select the correct time (usually approx. 6–12 h depending on the construct) when the expression was sufficient to measure FRET, the expressed proteins were localized as the endogenous wild type, and the functional activity was under control (see Fig. 3). Extending the time of the experiment (e.g. to more than 16–20 h after transfection for the KDELr-YFP) led to severe problems not only in the distribution of the expressed proteins but also in impaired transport function. Thus, cholera toxin was able to recognize the CFP or YFP-fused-KDELr 6–8 h after transfection (Fig. 3a,c) but was not able to enter the structures labeled by KDELr-fusion proteins after 16 h of expression (Fig. 3b,d; big arrows). In these cells CTX still bound to areas of the Golgi and exhibited only a low expression of the KDEL-receptor (Fig. 3b,d; small arrow).

2. FRET–MMM: sensitized emission and acceptor photoinactivation

The MMM experimental set-up is sketched in Fig. 1b. A mode-locked titanium:sapphire laser (Mira 900-F, Coherent, Palo Alto, CA) was used to provide 140 fs-pulses at a repetition rate of 76 MHz with ~2 W average power. Its infrared beam (λ = 800 nm) was expanded by a zoom telescope and passed through a color glass filter (OG550, Schott, Mainz), which was used to remove residual emission in the visible spectrum. By a rotating microlens disc the IR beam was split into ~30 single beamlets which entered the microscope and generated an array of high resolution foci on the sample (Bewersdorf et al., 1998; Straub and Hell, 1998a,b). As microscope an inverted Leica DM-IRB with oil immersion objective (Leica PL APO, 100x, NA1.4) was used. Fluorescence light passed a dichroic mirror and was detected by a CCD Camera (UltraPix Si250, LSR AstroCam, Cambridge, UK) or, alternatively, could be observed directly by the eye. In addition to an infrared cutoff filter (3 mm BG39, Schott) suitable bandpass and edge-filters were used as emission filters. Cells were kept in a temperature controlled superfusion microscope chamber at 37 °C during all MMM-experiments (Straub et al., 2000).

Using the mercury lamp of the Leica DM-IRB fluorescence intensity of CFP- and YFP-fusion proteins was measured before and subsequent to the MMM-experiments (CFP: 436/7 excitation-, 485/20 bandpass emission filter, YFP: 540/25 bandpass excitation-, 590 nm longpass emission
filter). Direct excitation of YFP by two-photon excitation at $\lambda = 800$ nm was negligible. If CFP and YFP are within $< 6$ nm, excitation of the CFP-moiety produces a mixture of cyan ($\lambda = 470$ nm) and yellow light ($\lambda \approx 525$ nm), as opposed to only cyan fluorescent light when there is no acceptor nearby any more after bleaching. FRET was estimated by comparing MMM images recorded with 485/20 bandpass and 550 nm long-pass (in combination with the IR cut-off filter effectively making up a 575/50 bandpass) immediately before and after bleaching of the live cell.

**Fig. 4**

ARF1-CFP (D)  KDELr-YFP (A)

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**Fig. 5**

Two photon:

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Figs. 4 and 5.
without any further readjustment of the sample. The YFP-component was bleached by 530 nm Ar-Kr-ion laser light (INNOVA 70C Spectrum, Coherent), applying 0.05 mW in each of the ~30 foci. At $\lambda = 530 \text{ nm}$ CFP (max. excitation: ~440 nm) bleaches only negligibly while more than 95% of the YFP (max. excitation: ~515 nm) bleaches within 3 min. Constancy of the z-position of the sample to ~100 nm was controlled by a capacitive sensor. Samples that changed shape during bleaching were disregarded. The FRET efficiency $E$ is obtained directly from the donor fluorescence intensities in the area of interest before ($D_{ab}$) and after ($D_{ah}$) acceptor bleaching, respectively. For perfect bleaching it holds that $E = 1 - D_{ah}/D_{ab}$. For imperfect bleaching, the bleaching efficiency, as determined from a comparison of the mercury lamp images of the YFP-distribution before and after bleaching, has to be taken into account. Time-dependence of FRET in a single cell has to be estimated from changes in sensitized emission (enhanced acceptor emission) upon two-photon excitation using the 550 nm longpass filter, because acceptor bleaching is not applicable under these conditions (Majoul et al., 2001).

Examples of the acceptor photobleaching and dequenching of the donor are given in Figs. 4 and 5. This pair of interacting CFP-/YFP-fusion proteins (ARF-CFP/KDELr-YFP) starts to reveal FRET upon cholera toxin transport. The example of intracellular distribution of $E$ for the pair ARF1-CFP/KDELr-YFP obtained 30 min after initiation of CTX uptake is calculated in Fig. 5. We selected here the combination of cytosolic donor ARF-CFP and the transmembrane acceptor KDELr-YFP. It is obvious that the cytosolic protein can meet the trans-membrane receptor only near the Golgi membrane. This is exactly where the maximum of $E$ was detected. After subtraction of the background $E$ can be calculated for each pixel. The results show that FRET–MMM allows for accurate and quantitative mapping of protein-protein interactions not only in living cells but also in subcellular compartments like the Golgi.

3. Spectrofluorimetric control

FRET between CFP- and YFP-fusion proteins is mainly dependent on their proximity. In overexpressing cells the unspecific stochastic interactions induced by very high local density of CFP-/YFP-fusion proteins may create an artificial FRET-signal, indicating that these cells cannot be used. Normally the interaction time of an unspecific pair for CFP-/YFP-fusion proteins should be significantly shorter than the interaction time displayed by a single pair of CFP-/YFP-fusion proteins undergoing specific interaction. To measure sensitized emission complementary to FRET–MMM data we used the same preparations in ‘bulk cell experiments’ with the spectrofluorimeter Fluoromax-2 (ISA, Jobin-Yvon Instr.) as a control. CFP was excited at $\lambda_{ex} = 425$ nm, and fluorescence emission was measured between 450 and 600 nm. For comparison, YFP was excited separately at $\lambda_{ex} = 498$ nm, its fluorescence was measured between 510 and 600 nm.
For time-dependent analyses of interactions between CFP- and YFP-fusion proteins, the culture plate was divided into segments and the cells from different segments were examined by spec-

![Fig. 6 Transmembrane FRET](image)

**Fig. 6** Transmembrane FRET

For time-dependent analyses of interactions between CFP- and YFP-fusion proteins, the culture plate was divided into segments and the cells from different segments were examined by spec-

**Fig. 7**

![Fig. 7](image)

**Fig. 7**

For time-dependent analyses of interactions between CFP- and YFP-fusion proteins, the culture plate was divided into segments and the cells from different segments were examined by spec-

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trofluorimetry before and after toxin application. We observed a good agreement between bulk cell experiments and single cell FRET–MMM measurements.

As already mentioned, the physiological validity of FRET-measurements in living cells expressing a given pair of CFP-/YFP-fusion proteins is strengthened if the observed FRET-signals are obtained in response to a trigger and compared with other pairs of CFP-/YFP-fusion proteins that stay unchanged. Cholera toxin induced FRET was recorded for the pairs KDEL-receptor-CFP/KDEL-receptor-YFP, KDEL-receptor-YFP/ARF-GAP-CFP, and KDEL-receptor-YFP/ARF1-CFP. In contrast, the FRET-signal of the pair p23-CFP/KDEL-receptor-YFP was elevated in the ground state and decreased significantly 30 min after initiation of CTX uptake. FRET between the pair p23-CFP/ARF-GAP-YFP remained almost unaffected by the same trigger over the duration of the experiment (Majoul et al., 2001).

These results underline the physiological importance of the data obtained by our experimental set-up.

4. Transmembrane FRET

A number of resident Golgi proteins represent type-I transmembrane proteins (p23 and p24 family members, Gommel et al., 1999). These proteins have a short cytoplasmic tail known to be essential for their physiological function. Therefore, p23- and p24-fusion proteins could be constructed only in a way that the CFP-moiety was linked to the N-termini of these proteins and located inside the Golgi lumen (Blum et al., 1999). As the Golgi pH is approximately 6.45, we used CFP attached to the N-termini within the Golgi (and not the YFP, whose spectrum is strongly pH-sensitive). As our aim was to study potential interactions between trans-membrane Golgi p23/p24 proteins (lumenal CFP) and the KDEL-receptor, ARF-GAP and ARF1 (cytosolic YFP) the question was raised whether trans-membrane FRET can be measured. The feasibility of measuring FRET across the Golgi membrane was first tested by expressing a control construct inserted into the Golgi membrane in a way that its N-terminal CFP-moiety was located in the Golgi lumen, while its C-terminal YFP-moiety was in the cytoplasm. Trypsin digestion experiments confirmed that the created transmembrane protein was properly localized in the membrane, i.e. that the fluorescent moieties were expressed on the different sides of the Golgi membrane (details in Majoul et al., 2001, and in ‘supplementary material’ thereof, including the theoretical consideration of the trans-membrane FRET). In cells expressing trans-membrane FRET control construct (FCC) the two-photon excitation of the (lumenal) CFP-moiety results in sensitized emission of the cytoplasmic YFP-moiety. Increased donor fluorescence after acceptor photobleaching confirmed that FRET can be measured across the Golgi membrane (Fig. 6). Based on these experimental data we performed FRET-proximity analyses of cells expressing the CFP- and YFP-fusion proteins mentioned above. We were able to record the transmembrane FRET for the following pairs of proteins: p24-CFP/KDEL-receptor-YFP, p24-CFP/ARF-GAP-YFP, p24-CFP/ARF1p, p23-CFP/KDEL-receptor-YFP, and p23-CFP/ARF1p-YFP. For the pair of p23-CFP/KDEL-receptor-YFP we observed time-dependent changes of the transmembrane FRET (sensitized emission) induced by cholera toxin. There is a good correlation between the transmembrane FRET-signal on the single cell level and the data obtained by spectrofluorimetry. Transmembrane FRET has also been measured between Cy3-labeled CTX-A-subunits and the KDEL-receptor-CFP. Interestingly, FRET was not observed at an earlier time-point (5 min) at which the CTX-A-Cy3 had been already taken in by cells but was not yet transported to the KDEL-receptor-CFP in the cis-Golgi (Majoul et al., 2001).

5. Outlook

Recent developments of the technology — generation of more stable fluorescent probes, discovery of a green fluorescent protein (GFP) and its genetically encoded spectral variants, introduc-
tion of DsRed and other fluorescent molecules — created a strong basis for a more detailed application of spectroscopy and microscopy in basic research, diagnostics and medicine. Here we introduce a successful application of FRET–MMM using it to monitor protein–protein interactions in living cells indicating that the ‘ruler’-principal (Fig. 7) can also be applied under near-physiological conditions. A major advantage of multifocal multiphoton microscopy in this application is the selection of a specific 1 µm thin plane of observation, which greatly enhances the reliability of the data. In addition, photostress was confined to the plane of observation and the time scale of the analyzed intracellular transport processes was in the range of minutes. It is nevertheless clear that the detection of intracellular processes by FRET is limited by the duration of the analyzed cellular processes. Clearly, the recorded process should not be shorter than the time of image acquisition and the mobility of the donor and the acceptor molecules should not influence the acceptor photobleaching. At the moment temporally extended measurements of comparatively fast biological processes, such as neurotransmitter release, signal transduction, trans-membrane translocation, rapid steps of vesicular transport, and other fast cellular processes are hampered by the rate of image acquisition. An alternative to FRET–MMM acceptor photobleaching is the fast ($\tau_D \sim 0.1–10$ ns) measurement of the donor life time. Direct life time measurements would be an ideal tool for cellular applications (Straub and Hell, 1998a) with the appearance of even better CCD cameras on the market; alternatively other set-ups such as multiphoton microscopy with time-correlated single-photon counting (Schönle et al., 2000) are also under development. Monitoring of the donor life time using picosecond-gated or modulated cameras and time-correlated photon counting used for three-dimensional analyses of intracellular FRET can provide us with a new tool to study live cell processes not accessible to earlier analyses thus acquiring biologically invaluable information. Alternatively, new bleach-resistant chromophores (e.g. Griffin et al., 1998) or spectrally novel fluorescent proteins (Matz et al., 1999) may help us to counteract the bleaching problems that occur under continuous image acquisition. Even more recent developments in microscopy, such as Ultrafast Dynamics Microscopy by STED (Klar et al., 2000; Dyba et al., 2000) may well extend our possibility to analyze intracellular processes and to contribute to a higher level of understanding of live cell function.

References


