Fixation, Mounting and Sealing with Nail Polish of Cell Specimens Lead to Incorrect FRET Measurements using Acceptor Photobleaching

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Key Words

FRET • ICln • Protein interaction • Photobleaching

Abstract

Fluorescence resonance energy transfer (FRET) is a technique used for the study of functional interactions between molecules. The intimate vicinity between two fluorescent molecules (FRET-pair; donor and acceptor) allows for an energy transfer, which can be directly calculated as the so called FRET efficiency. This technique is used in fixed as well as living cells. Here we show first, measured by the FRET technique, that the ICln ion channel is transposed from the cytosol towards the cellular membrane in HEK cells after swelling, and second, that the calculation of the FRET efficiency by de-quenching the donor cyan-fluorescent-protein (CFP) emission due to acceptor-photobleaching leads to erroneous estimate of the FRET efficiency in fixed, mounted and sealed specimens. The acceptor photobleaching leads to a modification of the donor cyan-fluorescent-protein, which shows then a strong emission, thus mimicking functional interaction between CFP (donor) and yellow-fluorescent-protein (YFP; acceptor).

Moreover, the procedure of acceptor photobleaching masks physiological (non random) interaction between molecules within the fixed, mounted and sealed cell. We show that no artifactual CFP modifications arise when using the acceptor photobleaching technique under in vivo conditions, and we offer strategies to minimize erroneous FRET efficiency calculations if cells need to be fixed.

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Introduction

The cytosol to membrane transposition of the mammalian ion channel ICln [1-3] is triggered by cell swelling [4, 5], as shown in several different cell systems [6-9]. We have shown this transposition by using Western-blot as well as fluorescence resonance energy transfer (FRET) [8]. FRET, as a measure for functional interaction between proteins in living or fixed cells, can be determined by de-quenching the donor CFP emission after acceptor YFP photobleaching [8, 10-12]. This method allows the direct calculation of the FRET efficiency, but the photodestruction of the acceptor does not allow repeated measures with the same sample. An alternative

method to detect FRET is the sensitized emission technique. In contrast to the former, this is a non-destructive method, which leads to the calculation of FRET indices, however, this method needs the consideration of a substantial number of correction parameters. The quantification of these parameters can be difficult and, moreover, can vary in an unpredictable way from experiment to experiment [13, 14]. The acceptor photobleaching approach has the advantage of being a faster and more direct method to detect FRET.

After having used this technique in living cells [8] we set out to test this technique in fixed cells to investigate the ICln affinity for different membrane domains, as lipid rafts. Measuring FRET in fixed samples should be more reliable and accurate, since (i) the mobility of the respective molecules (donor and acceptor) within the specimen should be abolished, (ii) the time course can be recorded more accurately. As we show here, after fixing, mounting and sealing the cells already under control conditions, where no FRET efficiency (FRET_{eff}) should be expected, a high FRET_{eff} could be calculated. Most importantly, this high FRET_{eff} did not further increase under conditions where a significant cytosol to membrane shift of ICln should occur, indicating that the fixing, mounting and sealing not only produces an artefactual FRET_{eff} determined by acceptor photobleaching approach, but, in addition, masks the physiological transposition of ICln measured *in vivo*. Therefore we set out to scrutinize these effects in greater detail, which, if leading to systematic erroneous FRET_{eff} calculations, could be of utmost interest in the era of proteomics or interactomics, since FRET_{eff} calculations are a standard method for measuring protein/protein interactions. Indeed, we show here that under commonly used experimental conditions, YFP bleaching (used in the acceptor photobleaching approach) can affect FRET measurements, and consequently lead to wrong calculations of FRET_{eff} and therefore lead to serious and systematic misinterpretations of the respective data. However, the experiments summarized here do also allow to indicate strategies by which the erroneous FRET_{eff} calculations can be minimized, if the specimens need to be fixed prior FRET measurements.

Materials and Methods

Cell culture

HEK-293 cells were cultured in minimal essential medium (MEM, Sigma M5650) containing 10% fetal bovine serum. The day before transfection, cells were seeded on a glass coverslip (12 mm diameter) in a 24 multiwell plate.

Transfection, fixation and mounting of the cells

Cells were transiently transfected with the calcium phosphate method with pEYFP-N1, pECFP-N1, pIRES2-EGFP (Clontech), or with a pcDNA3.1 vector containing an ECFP-linker (coding for GGTG)-EYFP tandem construct for the expression of the CFP-YFP fusion protein [15]. In the case of the ICln transposition experiments, HEK-293 cells were transfected with pECFP-C1-hICln (expressing a fusion protein of CFP and human ICln [8]) and pEYFP-Mem (Clontech). 48 hours after transfection, cells were exposed to a isotonic (NaCl 90 mM, KCl 5 mM, MgCl_2 2 mM, CaCl_2 2 mM, HEPES 10 mM, glucose 5 mM, mannitol 90 mM, pH 7.4) or hypotonic solution (NaCl 90, KCl 5 mM, MgCl_2 2 mM, CaCl_2 2 mM, glucose 5 mM, mannitol 10 mM, HEPES 10 mM, pH 7.4) for 20 minutes before fixation. In all the other experiments, cells were directly fixed 48 hours after transfection with 4% paraformaldehyde (PFA) in PBS (pH 7.4) for 10 min at room temperature, then washed three times in PBS and afterwards coverslips were mounted in Mowiol (polyvinyl alcohol 4-88; Fluka), DPX (distyrene plasticiser xylene; without dehydration of the specimen) or 90% glycerol in PBS and, unless otherwise stated, sealed with nail polish or paraffin as indicated. In some experiments, transfected cells (seeded on a 35 mm glass-bottom dish) were kept in PBS after fixation. After fixation and mounting, specimens were kept at 4°C in the dark, until images with the confocal microscope were acquired.

The experiments with the nail-polish ingredients isopropanol, toluene, and ethyl-acetate were performed by including 30% of the substance in mowiol mounting medium or, as in the case of nitrocellulose, by sealing the mowiol mounted coverslip with 7% (w/w) nitrocellulose dissolved in ethyl-acetate. The specimens were observed 1 day after fixing and mounting.

For the *in vivo* experiments, cells were seeded on a 35 mm glass-bottom dish, transfected, and after two days, imaged at the confocal microscope, after replacing culture medium with a Phenol red-free MEM medium (Sigma M3024), buffered with 10 mM Hepes, pH 7.4 (34 mM of mannitol was added in order to keep the osmolality at 290 mOsm). For the *in vivo* ICln transposition experiments, coverslips with transfected cells were transferred to the FCS-2 chamber (Bioptechs, Inc.) and mounted on the inverted microscope. Cells were kept 5 minutes in isotonic solution, images were acquired, and then the extracellular solution was changed for the hypotonic one and images were acquired again after 10, 15 and 20 minutes.

Confocal microscopy and FRET calculation

All images were acquired at room temperature with a Leica DM-IRE2 inverted microscope (TCS SP2 AOBS scanhead), equipped with an argon laser and a 63X oil immersion objective. CFP was excited with a 458 nm laser beam and the emission window was set at 465-505 nm; YFP was excited with a 514 nm laser beam (514LB) and the emission window was set at 525-600 nm. In both cases images were acquired with a 512x512 resolution, line average 2, 8 bit. Unless otherwise stated, photobleaching of YFP was performed with 4 sequential illuminations (4 frames, 1024x1024 resolution, line average 1) of the selected cell with 514LB (setting the AOTF at 100%), after zooming at 10x. All experiments were performed using 50% of the full

power laser unless otherwise stated. FRET efficiency was calculated from the ratio of the CFP fluorescence evaluated before (CFP_{pre}) and after (CFP_{post}) photobleaching, using the following formula:

$$FRET_{efficiency} = 1 - \frac{CFP_{pre}}{CFP_{post}} \quad (i).$$

Equation (i) is described in Ritter et al. [8]. Throughout the text the FRET efficiency (FRET_{eff}) is given in % according equation (ii):

$$FRET_{eff} = FRET_{efficiency} \cdot 100 \quad (ii).$$

The sensitized emission (F_{sen}) was calculated as described in vanRheenen et al. [16]. The magnitude of cross-talk between donor and acceptor was estimated by the parameters α , γ , δ and β . These correction factors are defined as:

$$\alpha = \frac{M_{Dy}}{M_{DAy}} \quad \gamma = \frac{M_{IAy}}{M_{DAy}} \quad \delta = \frac{M_{Dy}}{M_{IAy}}$$

(indices y indicate that the measured fluorescence was determined in samples expressing only YFP with the same acquisition parameters used throughout the experiments).

$$\beta = \frac{M_{IAc}}{M_{DAc}}$$

(indices c indicates that the measured fluorescence was determined in samples expressing only CFP with the same acquisition parameters used throughout the experiments). M indicates the measured fluorescence (minus background) in the regions of interest (ROI). Donor excitation with donor emission is indicated as M_D, donor excitation with acceptor emission is indicated as M_{IA}, and acceptor excitation with acceptor emission is indicated as M_{DA}.

F_{sen} was obtained according to the formula:

$$F_{sen} = \frac{M_{IA} - M_D\beta - M_{DA} \cdot (\gamma - \alpha\beta)}{1 - \beta\delta} \quad (iii)$$

The NFRET indices were calculated according Xia & Liu [17] in ROIs where a clear YFP-mem signal was present:

$$NFRET = \frac{F_{sen}}{\sqrt{M_D \cdot M_{DA}}} \quad (iv)$$

E_{app} was calculated according Zal & Gascoigne [14] in order to be able to compare acceptor photobleaching with sensitized emission:

$$E_{app} = \frac{NFRET}{NFRET + G} \quad (v)$$

where

$$G = \frac{F_{sen pre} - F_{sen post}}{M_{D post} - M_{D pre}} \quad (vi)$$

Pre and post indicate measurements before and after acceptor photobleaching. For our acquisition settings G was 1.02 ± 0.07 (n=8).

FRET efficiency and NFRET images were obtained by applying equations (i) or (iv) in a pixel-by-pixel way. In order to

suppress excessive noise in dim parts of the images, a mask was applied as described in [16].

Salts, chemicals and drugs

All salts, chemicals and drugs used were of *pro analysis* grade. Two different nail-polish commercial brands were used: Riveel (components: butyl acetate, ethyl acetate, nitrocellulose, phthalic anhydride/trimellitic anhydride/glycol copolymer, acetyl tributyl citrate, isopropyl alcohol, stearalkonium hectorite, camphor, citric acid; colour 10) and Broadway (components: butyl acetate, ethyl acetate, nitrocellulose, acetyl tributyl citrate, adipic acid/neopentyl glycol/ trimellitic anhydride copolymer, isopropyl alcohol, acrylates copolymer, stearalkonium bentonite, styrene/acrylates copolymer, benzophenone-1, mica, algae extract, citral; colour 8).

Statistical analysis

All values are given as mean ± SEM. Data were tested for statistically significant differences by the Student's t-test or by ANOVA, where applicable. A statistically significant difference was assumed at p<0.05.

Results

A hypoosmotic swelling of the cells leads to a significant transposition of ICln from the cytosol towards the cellular membrane in different cell lines as well as rat kidney tubular cells [6-9]. This transposition can reliably be evidenced by using Western-blot or FRET measurements in the living, i.e. *in vivo* cell system. One way for doing the latter kind of experiments is to use one chromophore, the donor (CFP), attached to the investigated protein (ICln in our case) and use the respective acceptor (YFP) attached to a protein-signal for posttranslational palmitoylation that targets YFP to cellular membranes (YFPmem) [8]. This experimental design is able to detect a transposition of the ICln protein towards the membrane, since after the transposition both chromophores are in an intimate vicinity to each other and therefore efficient FRET between them can occur.

Measurements of the membrane transposition of ICln after cell swelling in human embryonic kidney (HEK-293) cells under *in vivo* conditions

As shown in figure 1a, reducing the extracellular osmolality by 80 mosM leads to a significant increase of the NFRET signal at the cellular membrane, indicating a net membrane transposition of ICln after cell swelling. This experiment is well in agreement with former studies where we showed a similar transposition in fibroblasts. Indeed, as shown earlier [8], the same reduction of the extracellular osmolality in 3T3 mouse fibroblasts leads to

Fig. 1. Membrane transposition of ICln measured in *in vivo* conditions using HEK cells co-transfected with CFP-ICln and YFPmem vectors. a) Measuring the sensitized emission, a clear cytosol to membrane transposition of ICln can be seen. FRET was calculated as NFRET (see methods). b) The same experiments as shown in (a), however, the FRET signal was calculated as E_{app} as defined by Zal & Gascoigne [14] (see methods). Scale bar 10 μ m.

a very similar result as shown here for HEK cells (figure 1a). The NFRET calculations described above, are made by using the sensitized emission technique. However, this method needs the consideration of a substantial number of correction parameters. The quantification of these parameters can be difficult and, moreover, can vary in an unpredictable way from experiment to experiment [13, 14]. An alternative approach would be the use of the acceptor photobleaching technique. The latter has the advantage of being faster and allowing the direct measure of FRET efficiency, if compared to the sensitized emission approach. Furthermore we planned to use the acceptor photobleaching approach in fixed cells, since in fixed cells this approach should be even more reliable and accurate compared to *in vivo* conditions, since the mobility of the respective molecules within the specimen should be abolished.

Measurements of the membrane transposition of ICln after cell swelling in fixed, mounted and sealed cells

In order to do these experiments, both, CFP-ICln and YFPmem, were expressed in HEK-293 cells. After challenging the cells with a hypotonic medium, the cells were fixed, mounted and sealed, and then subjected to acceptor photobleaching in order to calculate $FRET_{eff}$. As shown in figure 2, reducing the osmolality of the bathing solution, leads to a slight increase of $FRET_{eff}$, indicating a transposition of ICln, however, this increase of $FRET_{eff}$ was, most surprisingly, not statistically significant. It is important to note that the same experimental procedure of lowering the extracellular osmolality did show a highly significant cytosol-to-membrane transposition as described above (figure 1a) and published by us [8, 9] and others [6, 7], if the measurements were made using living cells and NFRET was calculated. In order to exclude any possible errors by comparing NFRET (sensitized emission) and $FRET_{eff}$ determined by acceptor photobleaching (two techniques using different algorithms for calculating the respective signals) we set out to recalculate the NFRET signals by using an algorithm by which E_{app} can be obtained, a value that can be directly compared to the

Fig. 2. Hypoosmotic transposition of the ICln to the plasma membrane can not be observed in fixed, mounted and sealed cells. HEK-293 cells were transfected with ECFP-C1-hICln and EYFP-Mem and cells were exposed to a hypotonic stress in order to induce the ICln transposition from the cytosol to the membrane. Cells were fixed in 4% paraformaldehyde, mounted in Mowiol and sealed with nail polish before imaging. In the images the $FRET_{eff}$ calculated pixel by pixel using the formula reported in the experimental procedures, are given. In the graph, mean values obtained under isotonic conditions (control; n=8) and after reducing extracellular osmolality (hypo; n=9) are indicated.

Fig. 3. YFP bleaching does not create CFP-like YFP. YFP bleaching was done at 514 nm. Measurements were made in fixed, mounted (Mowiol) and sealed (nail-polish) HEK-293 cells expressing only the YFP protein. a) With grey (pre-bleaching) and red (postbleaching) lines the position for the fluorescence profiles given in the respective lower panels are indicated. Fluorescence is given in arbitrary units (au). Scale bar is 5 μm . b) Summary ($n=15$) of the experiments shown in (a). Note the different scaling of the y-axis.

FRET_{eff} values gained by acceptor photobleaching (see methods). As seen in figure 1b, the transformation of NFRET into E_{app} leads to a qualitatively indistinguishable result, therefore, the missing transposition seen in figure 2 is most likely due to the specific experimental setting. As furthermore shown in figure 2, already under control conditions - where a very low FRET signal should be expected, since the donor (CFP-ICln) and the acceptor (YFP-Mem) should be distributed mainly in different compartments - a substantial FRET signal can be detected. It seems that the use of fixed, mounted and sealed specimens could seriously hamper the use of acceptor photobleaching as a strategy to calculate FRET_{eff}. This is especially important, since fixing the cell specimens has undisputedly the advantage of desynchronizing the functional experiment (reducing the osmolality in our case) from the process of data collection, and furthermore is able to abolish the diffusion of the respective chromophores after photobleaching within the specimen, a circumstance, which should improve the sensitivity and accuracy of the technique. It is, however, known that cell fixation can lead to a changed optical behaviour of another chromophore, i.e. green fluorescence protein (GFP), whose emission is, however, decreased after fixing the specimen [18-20]. Therefore, we set out to test for, whether or not the process of cell fixation, mounting and sealing does interfere with the process of FRET_{eff} calculations by using the acceptor photobleaching approach.

Systematic measurements in order to elucidate a possible artefactual FRET signal by using the acceptor photobleaching

In order to test for a possible systematic error in calculating FRET_{eff} by using acceptor photobleaching in fixed, mounted and sealed cells, we used another set of chromophores. In one set of experiments we expressed in the cells the donor (CFP; C) or the acceptor (YFP; Y) alone. This approach should allow us to reveal any effect of the YFP bleaching procedure on the CFP or YFP molecules. In a second set of experiments we expressed

both, CFP and YFP, individually and simultaneously (C+Y). The latter leads to a stochastic distribution of both, donor (CFP) and acceptor (YFP) throughout the cytosol and therefore allows for revealing the random-FRET (or noise). Finally we used a CFP-YFP fusion protein (CY) as a positive control for FRET_{eff} [15]. We started out to test whether the acceptor photobleaching procedure leads itself to a modification of the donor or the acceptor, respectively, which could lead to artefactual FRET_{eff} calculations.

Acceptor photobleaching does not produce a CFP like YFP

Valentin et al. [11] reported that photobleaching of the YFP could lead to modified YFP molecules with CFP-like behaviour, whose signal adds to the donor (i.e. the CFP) signal. This signal is used for calculating the FRET_{eff} [8], thereby leading to an artefactual overestimation of the latter. Other reports, however, indicate that photobleaching of YFP does not produce CFP-like species that affect FRET measurements [10, 12]. Our experiments confirm these reports. As shown in figure 3, after expressing only YFP, the photobleaching of YFP

Fig. 4. Bleaching of YFP can lead to false-positive FRET_{eff} calculations by increasing the CFP signal in fixed, mounted and sealed cells. In HEK-293 cells a CFP/YFP fusion protein (CY), CFP and YFP as two independent proteins (C+Y), or CFP alone (C) were expressed. The FRET_{eff} which is calculated [8] using the de-quenching of the CFP signals, is given in the respective panels (a and b). a) Measurements are made 1 day after fixing, mounting and sealing the specimen. The respective FRET_{eff} is given, indicating that (i) the FRET_{eff} measured with the CFP/YFP fusion protein is smaller if compared to the 'living' condition depicted in (b), and (ii), false positive FRET_{eff} can be measured in conditions where no FRET_{eff} should be detectable at all (see text). In the insert an original experiment is shown in which only CFP was expressed, and hence no signal can be measured in the YFP channel. Scale bar is 10 μ m. b) Measurements are made using living cells. The insert shows an original set of images using the CFP/YFP fusion protein (CY), before and after YFP photo-bleaching using a 514 nm laser.

decreases, as expected, the signal in the YFP channel (YFP emission), however, no substantial emission can be measured in the CFP channel, therefore ruling out a CFP-like modification of YFP due to 514 nm bleaching.

Acceptor photobleaching leads to a modification of the optical behaviour of CFP only if measured in fixed, mounted and sealed cells, but not if measured in living cells

Since a photoconversion of YFP after acceptor photobleaching can be ruled out for the observed erroneous FRET_{eff} we focused on possible modifications of the CFP due to the photobleaching procedure. As shown in figure 4a, one day after fixing, mounting (Mowiol), and sealing the specimen with nail-polish, a substantial FRET_{eff}

Fig. 5. Modification of CFP in fixed, mounted and sealed cells is dependent on the laser energy and laser wave length. a) The laser energy is given in arbitrary units (au). In the grey area 100% laser energies were used, however, in order to reach higher energies multiple frames (exposition to laser light) were used (grey area). For 514 nm, the number of frames were 1, 4 and 10 respectively. For 633 nm, the number of frames were 4, 10, 20 and 40 respectively. For 543LB 1,4 and 10 frames were used. In order to decrease the energy of the 514 nm laser, the acousto-optical-tunable-filter (AOTF) was set to 50, 20, 10 and 5% (area left from the grey box). The arrow indicates the energy of 514LB used throughout the paper if not otherwise stated. b) A single 514LB photobleaching event is sufficient to effect a complete photoactivation of the CFP, since repetitive photobleaching events do not result in any further change in the CFP fluorescence (circles). The arrows indicate the 4 frame exposure with the 514nm photobleaching beam. In the absence of 514LB (triangles) the CFP emission is stable. Both measurements were made 1 day after sealing. The energy of the laser beam is given in (a). c) The 514LB is modifying the CFP in such a way that his emission between 470 and 505 nm is markedly increased.

could be calculated after acceptor photobleaching in cells expressing only CFP ($23 \pm 1\%$, $n=47$). In these experiments no YFP is expressed, however, the CFP was exposed to the laser-beam used for YFP bleaching. Equally important is that under these conditions the calculated FRET_{eff} of the CFP-YFP fusion protein (CY; used as a positive control for FRET_{eff}) is not statistically different

Fig. 6. Modification of CFP is dependent on the mounting and sealing conditions and the time. $FRET_{eff}$ was calculated after expressing CFP only. We found that using 90% glycerol instead of the Mowiol (polyvinyl-alcohol) mounting medium was able to delay the appearance of the 514LB induced photo-enhancement of CFP, but could not prevent it (light grey bars). If no sealing agent (nail polish) was used, no substantial photo-enhancement of CFP can be observed (black bars) after 2 hours, however, a small but significant increase of $FRET_{eff}$ could be measured after 4 days. Similar results could be obtained when paraffin was used instead of nail-polish. Fixed specimens mounted in Mowiol and sealed with paraffin reveal after 5 days a $FRET_{eff}$ of $2 \pm 1\%$ $n=4$. No artefacts could be detected also by keeping the specimens in PBS after fixing. This findings suggest the possibility that fixation of the cells with paraformaldehyde itself is not involved in generating the 514LB induced increase in CFP emission. The use of the mounting agent DPX creates itself artefactual CFP emission even in the absence of nail polish (the DPX value for the 4 day-group was not determined).

from the former ($22 \pm 1\%$, $n=32$). Accordingly, also the $FRET_{eff}$ calculated after expressing CFP and YFP individually ($C+Y = 20 \pm 1\%$, $n=39$) is not statistically different from CY (figure 4a). From these experiments can be concluded that it is not possible to make meaningful calculations of $FRET_{eff}$ one day after fixation, mounting, and sealing the specimen with nail-polish. However, when we repeated these experiments under in vivo conditions, we found exactly what should be expected. As shown in figure 4b, the non-FRET related increase of CFP emission after YFP bleaching is negligible when measuring FRET in living cells expressing only CFP ($C = 1 \pm 1\%$, $n=9$; figure 4b). In cells expressing CFP and YFP, a very small, but significant, $FRET_{eff}$ can be measured most likely due to a random interaction of CFP and YFP ($C+Y = 5 \pm 1\%$, $n=9$; figure 4b). However, the expression of the CFP-

Fig. 7. $FRET_{eff}$ calculations in fixed and Mowiol mounted cells. No nail-polish was used for sealing the specimens. Images were taken 2 hours (a) or 1 day (b) after mounting (Mowiol), and the respective inserts were made by expressing CY. YFP bleaching was done at 514 nm. Scale bars are 10 μm .

Fig. 8. CFP-ICln membrane transposition in fixed HEK cells kept in PBS. CFP-ICln and YFPmem transfected cells were exposed to the isotonic or to the hypotonic solution for 20 min, then fixed with PFA and kept in PBS (the mounting procedure was avoided). The acceptor photobleaching method was used to obtain the FRET efficiency. a) Cell images before and after the YFP photobleaching and $FRET_{eff}$ images for the isotonic and hypotonic conditions are shown. b) Avoiding the mounting and sealing procedure make the ICln translocation measurable again (iso, $n=13$ and hypo, $n=14$, $* = p < 0,05$). Scale bar 10 μm .

YFP fusion protein leads to a pronounced and significant increase of FRET to $46 \pm 3\%$ ($n=9$; $p<0.01$; figure 4b).

The artificial $FRET_{eff}$ in fixed, mounted and sealed cells calculated after using the 514 nm laser beam (514LB)

Table 1. CFP, GFP or YFP expressing cells were fixed and mounted in mowiol with (Mow + NP) or without (Mow – NP) the nail polish sealing. To compare the fluorescence between Mow + NP and Mow – NP samples, 5 to 13 images were acquired for each condition with the same instrument settings and the fluorescence (mean grey value) of each single cell was measured (n>100 in each condition). The % Δ fluorescence was calculated as $100 \cdot (F_{\text{mow-NP}}/F_{\text{mow+NP}} - 1)$ where $F_{\text{mow-NP}}$ or $F_{\text{mow+NP}}$ are the mean of fluorescence values from the + NP or the - NP condition. To obtain the extent of photobleaching the 514LB photobleaching protocol was applied as described in Experimental procedures and the photobleaching (%PB) was calculated as $(1 - F_{\text{post}}/F_{\text{pre}}) \cdot 100$, where FP is the tested fluorescent protein. % Δ PB represents the difference between %PB of the Mow + NP and Mow –NP condition.

FP	% Δ Fluorescence		% PB (514 LB)	
	(MOW + NP)- (MOW-NP)	MOW - NP	MOW + NP	% Δ PB (MOW+NP)- (MOW-NP)
CFP	-65.93	-0.59 $\pm$ 0.31 (n=9)	-31.70 $\pm$ 1.82 (n=47)	-31.11
GFP	-71.94	65.25 $\pm$ 2.01 (n=8)	16.47 $\pm$ 7.14 (n=6)	-48.78
YFP	-52.23	97.90 $\pm$ 0.27 (n=6)	78.98 $\pm$ 4.90 (n=7)	-18.92

for YFP photobleaching is most likely caused by modifying CFP in such a way that its emission at 465-505 nm (excitation at 458 nm) is increased. The 514LB-induced transformation of CFP depends on the energy of the 514LB (figure 5a). A similar behaviour of CFP could be observed after using the 543LB and the 633LB, although, especially in the latter case, the effect on CFP was less efficient (figure 5a). As shown in figure 5b, if a 514LB was used at an energy producing a maximal CFP signal (figure 5a), recurrent 514LB exposures of the specimens did not increase the CFP signal further, indicating that a single laser-beam photobleaching event is sufficient to alter the optical behaviour of CFP, which then remains stable. Our hypothesis that the 514LB enhances the CFP emission is further substantiated by the emission spectra shown in figure 5c. As shown, the 514LB used for YFP photobleaching increases the emission of the CFP fluorescence between 470 and 505 nm.

The described alteration of the optical properties does not seem to be restricted to CFP, since GFP expressed in HEK-293 cells, presents a similar behaviour. Since 514 nm is very close to the GFP excitation peak, the 514LB exposure results in the GFP photobleaching. The exposure to the 633LB (40 frames at 100% of laser power) causes a significant increase of the FRET_{eff} signal in GFP expressing cells fixed, mounted and sealed with nail polish. ($7 \pm 3\%$, n=7).

In the absence of nail-polish the modification of the optical behaviour of CFP by the 514LB is markedly attenuated

Performing the experiments in the absence of nail-polish alleviates the described problem (figure 6 and 7). As shown in figure 7a, if only CFP is expressed, 2 hours after mounting the specimen, no FRET_{eff} can be calculated ($C = 0 \pm 1\%$, n=17), and only a small value can be calculated after one day ($C = 2.0 \pm 0.3\%$, n=5; figure 7b). However, one day after fixing and mounting, and to a lesser extent 2 hours after fixing and mounting,

the random-FRET measured by expressing C+Y, is significantly higher, as compared to the *in vivo* condition (figure 4b and figures 7a and b). Paraformaldehyde fixation and polyvinyl-alcohol based mounting medium (Mowiol) obviously increases the random-FRET, and lower therefore the signal-to-noise ratio of the FRET_{eff} calculations. However, the real FRET_{eff} measured by the expression of the CY fusion protein (figure 7) is not significantly decreased, if compared to the *in vivo* condition (figure 4b). Identical results were obtained by employing two different brands of nail-polish, with similar but not identical composition. To identify which compound of nail polish was responsible for the described effect on CFP, we tested some of the most commonly used ingredients (i.e. nitrocellulose, isopropanol, ethyl-acetate and toluene) on fixed and mowiol mounted cells expressing CFP. Ethyl-acetate was the only substance that could partially reproduce the nail polish effect (FRET_{eff} = $5 \pm 1\%$, n=7).

Nail polish seems to affect not only CFP but also GFP and YFP behaviour. 514LB induced photobleaching was markedly decreased if nail polish was used to seal HEK-293 cells transfected with GFP or YFP (table 1). A similar effect was observed after exposing CFP to the 488LB, a wavelength that causes its photobleaching (% photobleaching = $85 \pm 2\%$, n=8 without nail-polish and $48 \pm 1\%$, n=9 with nail-polish, p<0,01). This suggests that, when nail polish is present, the exposure to a laser beam that is able to bleach GFP, CFP or YFP, is likely to generate two opposed phenomena: photobleaching and enhancement of the emission signal, which eventually sum and result in a decreased photobleaching. Moreover, we also noticed that all the chromophores we examined showed a significantly lower fluorescence level (without exposure to the 514LB) when nail polish was used (table 1).

In figure 6 we additionally show that the mounting conditions themselves are able to alter the FRET_{eff} calculation. Using glycerol instead of Mowiol does not change the magnitude of the 514LB induced CFP modi-

fication measured after 7 days, however, a delay in the onset of the problem can be evidenced. In fixed specimens kept in PBS without mounting and sealing, no artefacts could be detected 2 hours or 7 days after fixation ($\text{FRET}_{\text{eff}} = -1 \pm 1\%$, $n=4$ and $-1 \pm 1\%$, $n=4$, respectively, Fig. 7). This finding suggests the possibility that fixation of the cells with paraformaldehyde itself is not involved in generating the 514LB induced increase in CFP emission. It is important to note, that mounting the fixed cells in DPX without sealing, already substantially increases the artefactual FRET_{eff} after 2 hours, and should therefore not be used for these types of experiments.

On the base of these findings we measured the swelling induced translocation of ICln-CFP in fixed cells kept in PBS, the condition showing no significant CFP artefact even after 7 days from fixation. With this protocol we could evidence again in fixed cells a significantly different FRET_{eff} measured in isotonic compared to the hypotonic condition ($\text{FRET}_{\text{eff}} = -1.9 \pm 1.6\%$, $n=13$ and $\text{FRET}_{\text{eff}} = 2.9 \pm 1.4$, $n=14$, Fig. 8).

Discussion

In order to scrutinize a close and ‘non stochastic’ vicinity between different proteins we calculate FRET_{eff} after acceptor photobleaching. An alternative approach to determine FRET_{eff} is the sensitized emission technique. The acceptor photobleaching approach has the advantage of being a faster and more direct method to detect FRET_{eff} if compared to the latter. However, a possible downside for this technique is the fact that environmental factors (such as cell shape changes or diffusion of molecules and dyes) can decrease its reliability and the accuracy, if used in *in vivo* systems. A way to overcome these potential problems is using the acceptor photobleaching technique in fixed specimen, where the diffusional processes of donor and acceptor and changes of the cell shape induced by the photobleaching procedure are minimized, and therefore they do not substantially influence the FRET_{eff} calculation. This is particularly important when analysing interactions in restricted regions of the cells as the plasmamembrane. For these reasons acceptor photobleaching is often used in fixed cells. We investigated the cytosol to membrane transposition of the ion channel ICln under hypotonic conditions in specimens, which were fixed, mounted and sealed. However, doing the experiments in this way failed to reveal a statistically significant cytosol to membrane transposition of ICln, which, however, was clearly observed under *in vivo*

conditions. We speculated, that fixing, mounting and sealing could be the reason for the observed discrepancy. Indeed, we show here, by expressing single FRET-proteins or a FRET-pair fusion protein, that fixing, mounting and sealing the specimen not only leads to an artefactual FRET_{eff} calculation when no FRET_{eff} should be expected, but, moreover, abolishes the FRET_{eff} when it should be expected. These findings explain the results we obtained in fixed, mounted and sealed specimens when we investigated the membrane transposition of ICln after cell swelling.

The findings described in the present paper suggest that some of the components of commercial nail polishes and DPX and to a lesser extent of the mounting agents Mowiol and glycerol, could play a major role in the modification of CFP fluorescence after 514LB exposure. Ethylacetate is one of the nail polish component that can affect CFP fluorescence, but it's likely that more than one single organic compound is responsible for the effect of nail polish.

The 514LB induced artefact (causing an enhanced CFP signal) depends furthermore on the time lapsing from fixing, mounting and sealing to the moment when the measurements are performed. The latter seems especially important for the nail-polish-induced problem, since the dimensions of the specimen determine how fast the modifying components of the sealant diffuse throughout the sample and therefore provoke the problem. Nail polish affects also GFP and YFP fluorescence behaviour. If the photobleaching protocol uses a wavelength far enough from the excitation peak of the fluorescent protein (FP), an increased emission results. If the laser beam wavelength photobleaches the FP, the enhanced emission appears as a decreased photobleaching. This does not seem to be the only effect of nail polish on the GFP family members, since it seems to decrease the emission of GFP [19, 20], which is commonly used to monitor gene expression in cells. In addition, it was shown that paraformaldehyde fixation can decrease GFP emission [18]. In accordance with these reports, we could indeed verify that sealing our specimens with nail polish markedly decreased the basal emission signal (without exposure to the 514LB) of CFP, YFP and GFP. It is possible to hypothesize that the two different phenomena are strictly related: the 514LB (and possibly other wavelengths) exposure allows CFP to recover from a quenched condition induced by nail polish sealing. From this we can conclude that nail-polish should be avoided completely if fluorescence measurements are made using GFP, CFP or YFP. A good alternative seems to keep the fixed cells

in PBS. When we tested this condition, we were able to measure the ICln translocation also in fixed cells, but the FRET_{eff} (calculated with the acceptor photobleaching in fixed cells), and the FRET_{app} (measured with the sensitized emission in the *in vivo* condition), are different. This suggests that the method for measuring the FRET signal and the cell condition (*in vivo* or fixed) can affect the absolute values of FRET_{eff} but not the biological process under study.

In conclusion, we show that YFP-bleaching related -enhancement of CFP emission, which could lead to wrong calculations of FRET_{eff} and therefore dramatic misinterpretation of the respective data, could easily be prevented if FRET measurements are made under 'native' conditions. However, if fixing is necessary, keeping the specimens in PBS or mounting in Mowiol and doing the measurements within 1 day is an option, only if no nail

polish is used. Fixing and mounting, however, decreases the signal-to-noise ratio by increasing the random FRET signal.

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References

- Furst J, Ritter M, Rudzki J, Danzl J, Gschwentner M, Scandella E, Jakab M, König M, Oehl B, Lang F, Deetjen P, Paulmichl M: ICln ion channel splice variants in *Caenorhabditis elegans*: voltage dependence and interaction with an operon partner protein. *J Biol Chem* 2002;277:4435-4445.
- Furst J, Schedlbauer A, Gandini R, Garavaglia ML, Saino S, Gschwentner M, Sarg B, Lindner H, Jakab M, Ritter M, Bazzini C, Botta G, Meyer G, Kontaxis G, Tilly BC, Konrat R, Paulmichl M: ICln159 folds into a pleckstrin homology domain-like structure. Interaction with kinases and the splicing factor LSM4. *J Biol Chem* 2005;280:31276-31282.
- Paulmichl M, Li Y, Wickmann K, Ackerman M, Peralta E, Clapham D: New mammalian chloride channel identified by expression cloning. *Nature* 1992;356:238-241.
- Furst J, Botta G, Saino S, Dopinto S, Gandini R, Dossena S, Vezzoli V, Rodighiero S, Bazzini C, Garavaglia ML, Meyer G, Jakab M, Ritter M, Wappler-Kornherr E, Paulmichl M: The ICln interactome. *Acta Physiol (Oxf)* 2006;187:43-49.
- Furst J, Jakab M, König M, Ritter M, Gschwentner M, Rudzki J, Danzl J, Mayer M, Burtscher CM, Schirmer J, Maier B, Nairz M, Chwatal S, Paulmichl M: Structure and function of the ion channel ICln. *Cell Physiol Biochem* 2000;10:329-334.
- Musch MW, Davis-Amaral EM, Vandenberg HH, Goldstein L: Hypotonicity stimulates translocation of ICln in neonatal rat cardiac myocytes. *Pflügers Arch*. 1998;436:415-422.
- Musch MW, Luer CA, Davis-Amaral EM, Goldstein L: Hypotonic Stress Induces Translocation of the Osmolyte Channel Protein pICln in Embryonic Skate (*Rajajelania*) Heart. *J. Exp. Zoology* 1997;277:460-463.
- Ritter M, Ravasio A, Jakab M, Chwatal S, Furst J, Laich A, Gschwentner M, Signorelli S, Burtscher C, Eichmüller S, Paulmichl M: Cell swelling stimulates cytosol to membrane transposition of ICln. *J Biol Chem* 2003;278:50163-50174.
- Tamma G, Procino G, Strafino A, Bononi E, Meyer G, Paulmichl M, Formoso V, Svelto M, Valenti G: Hypotonicity induces aquaporin-2 internalization and cytosol-to-membrane translocation of ICln in renal cells. *Endocrinology* 2007;148:1118-1130.
- Thaler C, Vogel SS, Ikeda SR, Chen H: Photobleaching of YFP does not produce a CFP-like species that affects FRET measurements. *Nat Methods* 2006;3:491; author reply 492-493.
- Valentin G, Verheggen C, Piolot T, Neel H, Coppey-Moisan M, Bertrand E: Photoconversion of YFP into a CFP-like species during acceptor photobleaching FRET experiments. *Nat Methods* 2005;2:801.
- Verrier SE, Soling HD: Photobleaching of YFP does not produce a CFP-like species that affects FRET measurements. *Nat Methods* 2006;3:491-492; author reply 492-493.
- Berney C, Danuser G: FRET or no FRET: a quantitative comparison. *Biophys J* 2003;84:3992-4010.
- Zal T, Gascoigne NR: Photobleaching-corrected FRET efficiency imaging of live cells. *Biophys J* 2004;86:3923-3939.
- Zimmermann T, Rietdorf J, Girod A, Georget V, Pepperkok R: Spectral imaging and linear un-mixing enables improved FRET efficiency with a novel GFP2-YFP FRET pair. *FEBS Lett* 2002;531:245-249.
- van Rheenen J, Langeslag M, Jalink K: Correcting confocal acquisition to optimize imaging of fluorescence resonance energy transfer by sensitized emission. *Biophys J* 2004;86:2517-2529.
- Xia Z, Liu Y: Reliable and global measurement of fluorescence resonance energy transfer using fluorescence microscopes. *Biophys J* 2001;81:2395-2402.
- Brewis N, Phelan A, Webb J, Drew J, Elliott G, O'Hare P: Evaluation of VP22 spread in tissue culture. *J Virol* 2000;74:1051-1056.
- Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC: Green fluorescent protein as a marker for gene expression. *Science* 1994;263:802-805.
- Wang S, Hazelrigg T: Implications for bicoid mRNA localization from spatial distribution of exu protein in *Drosophila* oogenesis. *Nature* 1994;369:400-403.