A fusion tag enabling optical marking and tracking of proteins and cells by FRET-acceptor photobleaching

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Summary
Combined time-lapse imaging with optical marking of fluorescent proteins (FPs) is a widely used method in studies of the dynamic behaviour of proteins, organelles and cell populations. Most of the approaches have specific limitations as they do not permit simultaneous observation of marked and non-marked molecules, require co-expression of two FP-tagged proteins or rely on oligomerizing FPs. Here we provide a strategy to overcome such limitations with a fluorescence resonance energy transfer-competent tandem fusion tag composed of two FPs. We combine optical marking by acceptor photobleaching with spectral imaging to discriminate between marked and non-marked molecules. Such ‘bleach-labelling’ may be employed in a broad range of studies for robust real-time tracking of proteins, organelles and cells.

Introduction
Understanding the dynamic behaviour of proteins, organelles and cell populations is a key challenge in the post-genomic era. Since the advent of genetically encoded probes yielding fluorescent proteins (FPs), the kinetics underlying labelled structures can readily be monitored in vivo. Widely employed methods in this regard combine time-lapse imaging with optical marking by photobleaching, photoactivation or photoconversion of FPs. Most of these techniques have specific limitations as they either do not permit simultaneous observation of marked and non-marked molecules, require co-expression of two FP-tagged proteins or rely on oligomerizing FPs. In fixed biological samples, the occurrence of fluorescence resonance energy transfer (FRET) between fluorophore labels is widely assessed through selective photobleaching of acceptor fluorophores. Here, the release of donor quenching upon acceptor destruction is monitored and provides a measure of FRET efficiency (Kenworthy, 2001). We reasoned that acceptor bleaching could also be employed in live-cell imaging to mark and subsequently to track structures labelled with FRET-competent FP pairs (Fig. 1A). To enable tracking, characteristic differences in their fluorescence emission spectra may be exploited to discriminate ‘bleach-labelled’ from ‘non-bleached’ molecules.

Materials and methods

Construct design
Standard recombinant DNA techniques, including PCR, were employed to clone vectors for ECFP-EYFP, ECFP-Venus and H2B-ECFP-Venus. ECFP cDNA was cloned from pECFP-N1 (BD Biosciences Clontech, Palo Alto, CA, U.S.A.), EYFP cDNA from pEYFP-N1 (BD Biosciences Clontech), Venus cDNA from pCS2 Venus (Nagai et al., 2002) and histone H2B cDNA from H2BGFP-N1 (Kanda et al., 1998). ECFP-EYFP and ECFP-Venus contain, from the N terminus, the following features (fluorophore sequence parts underlined; linker sequences italicized): ECFP-Linker-EYFP or Venus (MVSKGE[…]DEL[YK]-SGGGG-MVSKGE[…]DEL[YK]). H2B-ECFP-Venus contains H2B-Linker-ECFP-Linker-Venus (MPEPAK[…]KYTSAK-GGGG-MVKGKE[…]DELYK-SGGGG-MVKGKE[…]DELYK). For expression in mammalian cells, vectors comprise a cytomegalovirus promoter and a polyA signal (from the bovine growth hormone gene).

Cell culture, transfection and sample preparation for microscopy
COS-7 cells (African green monkey) were maintained in a humidified atmosphere at 37 °C and 7.5% CO₂ in Dulbecco’s modified Eagle medium supplemented with 10% fetal calf serum, 2 mm l-glutamine and 50 µM β-mercaptoethanol (all cell culture reagents from Gibco, Invitrogen GmbH, Germany). Cells grown to 70% confluency were transfected by a modified DEAE dextran/chloroquine protocol (see Supplementary material). Twelve hours post-transfection, cells were transferred to either glass-bottomed dishes (WillCo Wells B.V., The
Netherlands) or cover-slides. The following day, cells in glass-bottomed dishes were examined alive, whereas cells on cover-slides were fixed (15 min with 4% paraformaldehyde dissolved in phosphate-buffered saline, PBS) and mounted with Pro-Long anti-fading solution (Molecular Probes Europe B.V., The Netherlands).

**Microscopic visualization**

Fixed cells were visualized with a Leica TCS SP2 confocal scanner using an HCX PL APO 63×/1.32–0.6 Oil CS lens (Leica Microsystems, Mannheim, Germany). Images were acquired at the instrument settings specified in Fig. 1. Local acceptor photobleaching was performed with the 514-nm laser line in conjunction with the optical zoom option.

Live cell imaging was performed with a Zeiss LSM 510 Meta system using a C-Apochromat 63×/1.2 water-immersion lens (Zeiss, AIM, Jena, Germany). Fluorescence emission was collected with the spectral Meta detector usually in the 10-nm width mode (collecting from 450–550 nm) or by setting it up in a two-channel mode, collecting from 450–505 nm and 515–602 nm. The scanned emission range was λ<sub>EM</sub> = 505–680 nm for SP1 and λ<sub>EM</sub> = 450–680 nm for SP2 (monochromator slit width: 10 nm for excitation, 5 nm for emission). The equations used in identifying spectral components in SP1 of ECFP-EYFP and ECFP-Venus are given in the supplementary material to this paper.

**Results and discussion**

**Expression and characterization of FRET-competent tandem FP fusions**

We generated chimeric tandem FP molecules by fusing enhanced cyan fluorescent protein (ECFP) to either enhanced yellow fluorescent protein (EYFP) or Venus, a variant of EYFP (Nagai et al., 2002). The resulting fusion proteins, ECFP-EYFP and ECFP-Venus, were transiently expressed in COS-7 cells and their spectral features assessed by spectrophotometry. For each construct, fluorescence emission spectra (Fig. 1B) were recorded at two different excitation wavelengths (λ<sub>EX</sub> = 430 nm and λ<sub>EX</sub> = 490 nm) as the excitation wavelength. The scanned emission range was λ<sub>EM</sub> = 450–680 nm for SP1 and λ<sub>EM</sub> = 505–680 nm for SP2 (monochromator slit width: 10 nm for excitation, 5 nm for emission). The equations used in identifying spectral components in SP1 of ECFP-EYFP and ECFP-Venus are given in the supplementary material to this paper.
reveals that in both constructs, FRET causes the emission of similar numbers of photons per acceptor fluorophore. By contrast, ECFP-EYFP exhibits stronger emission of fluorescence from the donor than ECFP-Venus. From this we conclude that, despite similar FRET efficiency, a remarkably larger fraction of ECFP-EYFP molecules lacks properly folded FRET acceptors. This supports findings by Nagai et al. (2002) demonstrating that fluorophore maturation is more efficient in Venus than in EYFP. Hence, the course of the present study was continued exclusively with ECFP-Venus fusions.

In fixed ECFP-Venus transfectants, we selectively photobleached Venus within a predefined area by high-power illumination with the 514-nm laser line of a confocal microscope. Before and after bleaching, images were acquired at three different excitation and emission detection settings (as specified in Fig. 1C) to monitor changes in emission characteristics. As expected, bleaching of Venus leads to an increase in ECFP emission due to the release of donor quenching (Fig. 1D). The observed magnitudes of intensity change indicate that molecules having a bleached FRET acceptor can be distinguished spectrally from non-bleached ECFP-Venus fusions. Furthermore, intensities measured in channels D and F (Fig. 1D) show that sufficient information to enable such discrimination can be obtained through parallel multichannel image acquisition upon excitation at 458 nm. Thus, data recording at high frame rate becomes feasible as time-consuming sequential imaging modes can be avoided. Of note, intensities measured within the photobleached area (ROI2) are slightly different in channels F and A because the contribution of CFP emission is only detectable in F (Fig. 1).

**Optical marking of ECFP-Venus in live cells**

Next, we sought to evaluate FRET acceptor bleaching for marking ECFP-Venus molecules in live cells. The intracellular localization of ECFP-Venus resembles that of green fluorescent protein (GFP); uniform distribution of fluorescence is observed in the nucleus and throughout the cytosol, whereas ECFP-Venus is excluded from membranous structures such as mitochondria and endosomes (Fig. 2). We repetitively photobleached Venus at $\lambda = 514 \text{ nm}$ within a cytosolic region of a live transfectant and monitored local changes in the cell’s emission spectrum by parallel multichannel imaging. To discriminate between signals derived from marked and non-marked ECFP-Venus molecules, subsequent image processing involved the so-called emission fingerprinting algorithm (Dickinson et al., 2001) using reference spectra of non-bleached or fully bleached ECFP–Venus molecules recorded earlier. This approach revealed that ECFP-Venus exhibits very high intracellular mobility, as evident from a decrease of the ECFP/Venus intensity ratio in the photobleached area followed by an increase of this ratio in other cytosolic regions (Fig. 2 and supplementary movie). Within seconds after a local bleach pulse, optically marked molecules were detectable throughout the cytosol, and non-marked molecules quickly entered the photobleached region. By contrast, nucleocytoplasmic exchange occurred very slowly, indicating that the nuclear membrane represents a pronounced barrier for ECFP-Venus mobility (Fig. 2, supplementary movie). In transfectants subjected to repeated rounds of bleaching, the percentage of bleach-labelled molecules increased in a stepwise manner with every bleach pulse until the pool of non-bleached molecules was exhausted (Fig. 2).

In the case of ECFP-Venus, our approach shows a new possibility for labelling and subsequent real-time tracking of mobile proteins in live cells. Moreover, assessing the relative abundance of bleached and non-bleached molecules pixel-by-pixel renders data analysis insensitive to changes in sample localization and morphology.

**Use of ECFP-Venus as a protein fusion tag**

We selected human histone H2B as a model to explore whether ECFP-Venus can be fused to other proteins without disrupting their normal localization and function. The H2B-ECFP-Venus fusion protein was localized to the nucleus and decorated condensed chromatin of mitotic cells (Fig. 3). Cytosolic H2B-
ECFP-Venus was only detected in cells having extremely high expression levels. At different stages of the cell cycle, staining patterns observed for H2B-ECFP-Venus were identical to those of H2B-EGFP, its corresponding single-FP counterpart (Kanda et al., 1998). We subjected interphase nuclei of live H2B-ECFP-Venus transfecteds to the same acceptor bleaching procedure as for cytosolic ECFP-Venus. The data acquired show that the vast majority of optically marked H2B-ECFP-Venus molecules remained localized to the bleached region throughout the course of the experiment (Fig. 3, supplementary movie). This immobile fraction appears to be stably incorporated into chromatin. In addition, a small pool of H2B-ECFP-Venus molecules was observed that exhibits high mobility and diffuses freely within the nucleus. This was evident from the initial small increase in the ECFP signal within the non-bleached area and from a decrease in the signal intensity ratio in the bleached area after each round of bleaching clearly visible in Fig. 3(C) at least in the first 100 s of the experiment. This can be explained by diffusion of non-bleached molecules into that area and diffusion of bleached molecules out of it. In the non-bleached area the ratio signal cannot increase in a step-wise manner as the signal there is strongly 'diluted' with non-bleached molecules. However, there is still a small increase in the raw ECFP signal within the first 100 s. Our finding confirms observations recently reported from fluorescence correlation spectroscopy studies on the mobility of YFP-tagged histone H2B (Wachsmuth et al., 2003). Taken together, these results demonstrate that multiple known aspects of histone H2B function can readily be reproduced with H2B-ECFP-Venus despite the size of its ECFP-Venus tag. As the same may also apply to other chimeric proteins containing ECFP-Venus, this tagging strategy could potentially be widely employed to design traceable FP probes. Support for this notion comes from a recent study that describes normal localization of fusion proteins containing structurally similar tandem FP protein tags (Fradkov et al., 2002).

Cell labelling

The percentage of ECFP-Venus-tagged molecules that are bleach-labelled upon local high-power illumination at $\lambda_{ex} = 514$ nm varies depending on exposure conditions. We sought to exploit this to provide a number of cells with a unique mark that would subsequently enable cell tracking. To test the feasibility of this approach, we bleach-labelled several nuclei of H2B-ECFP-Venus cells differently by exposing areas to bleach pulses of different duration. This was achieved by scanning the bleach areas iteratively 100, 200, 400 and 600 times. Images then taken show that within these areas, the composition of bleach-labelled and non-bleached ECFP-Venus tags was indeed unique for every cell (Fig. 4). As such labels are expected to persist for at least several hours, this strategy may be applied to monitor multiple cells simultaneously. Experiments to examine the feasibility of this approach for cell tracking in developmental biological studies are underway. Although the ECFP/Venus signal intensity ratio will change over time due to protein turnover, unique spectral characteristics generated by bleach-labelling should permit an identification of individual cells for many hours.
This method can be exploited for whole-cell labelling using cytosolic constructs, but organelle labelling and tracking should also be feasible.

**Conclusion**

In the current study, we present an alternative approach for real-time optical marking and tracking *in vivo* that is based on fusion proteins containing the tandem FP tag ECFP-Venus. Unlike other methods of FP photobleaching and photoactivation, the imaging approach described permits simultaneous observation of optically marked as well as non-marked molecules throughout an experiment. Our method not only extends but can also simplify the recently established FLAP (fluorescence localization after photobleaching) technique, which involves co-expression of CFP- as well as YFP-tagged versions of the same protein species (Dunn *et al*., 2002). In FLAP, YFP fusions are selectively photobleached whereas CFP fusions serve as a reference to enable tracking of bleach-labelled molecules by image differencing (Dunn *et al*., 2002; Gerlich *et al*., 2003; Zicha *et al*., 2003). Unlike the originally described FLAP method, fluorophores required for protein tracking reside on the same polypeptide chain in ECFP-Venus-tagged proteins. Thus, the stoichiometry of ECFP and Venus is fixed and carefully controlled co-expression of two fusions is not required. ECFP-Venus tags behave like FPs that change emission characteristics in a light-controllable manner. In this context, spectral imaging is simply used to discriminate between both states. However, one has to take into account that the relative large size of the ECFP-Venus label (55 kDa) can have negative effects on the mobility of the protein of interest. Switching of spectral characteristics can also be achieved with DsRed from *Discosoma striata* and with Kaede, a recently isolated FP from *Trachyphyllia geoffroyi* (Marchant *et al*., 2001; Ando *et al*., 2002). However, like many other coral-derived FPs, both DsRed and Kaede have an intrinsic tendency to tetramerize that severely limits their use as fusion tags (Wall *et al*., 2000; Ando *et al*., 2002; Verkhusha & Lukyanov, 2004).

Our ECFP-Venus construct could also be sensitive to dimerization, but neither ECFP nor Venus have the tendency to dimerize and the high FRET efficiency we have measured suggests otherwise. The pH sensitivity of the tandem construct was not examined here, but CFP and Venus (Nagai *et al*., 2002) alone are both relatively insensitive to pH changes compared with other FPs such as YFP or EGFP. Interestingly, under low oxygen conditions, spectral photoconversion is even feasible with the widely employed GFP from *Aequorea victoria* (Elowitz *et al*., 1997; Jakobs *et al*., 2003). As environmental requirements for such photoconversion are incompatible with normal cell maintenance, GFP cannot be universally employed as an ‘optical highlighter’. ECFP-Venus overcomes these limitations, as it does not promote fusion protein aggregation and bleach-labelling.

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**Fig. 4.** Differential marking of cells by variation of bleaching conditions. Rectangular areas within nuclei of H2B-CFP-Venus transfectants were differentially marked by varying the duration of high-power illumination at 514 nm (numbers specified in C refer to rounds of bleaching per nucleus). Colour-coded images show cells before (A) and after (B) repetitive bleaching. The corresponding emission spectra (normalized) obtained from regions outlined in B are shown in D and E. A and B show the overlay of the CFP and the Venus channel after unmixing the spectral images. Visual discrimination of cells is facilitated if results are presented as a ratio image of the CFP/Venus intensities (C).
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References


Supplementary material

The following supplementary material is available online.

Transfection of COS-7 cells: modified DEAE dextran method

Cells grown to 70% confluence on dishes 10 cm in diameter were transfected by a modified DEAE dextran/chloroquine protocol: medium was exchanged for 4 mL PBS to which 1 mL of transfection solution (DMEM medium. 2% FCS, 10 μg circular plasmid DNA, 0.9 mmol chloroquine and 0.3 mg mL⁻¹ DEAE-Dextran) was added. After 3 h at 37 °C, transfection solution was aspirated and cells covered for 2 min with DMSO solution (DMEM medium with 2% FCS and 10% DMSO). Finally, the DMSO solution was exchanged for normal DMEM containing all supplements listed above. Twelve hours later, transfectants were transferred to either glass-bottomed dishes (WillCo Wells B.V., The Netherlands) or cover-slides.

Microscopic visualization

Time series of live cells were acquired at varying rates (0.3–2 Hz) and different durations (1200–2000 timepoints) usually using a pixel time of 1.6 μs and a pinhole setting of 1 airy unit. Excitation was with the 458-nm line, bleaching with the 514-nm line of the argon laser. For efficient bleaching, the laser tube current was set to 85% at 514 nm and the LSM was operated in bi-directional scanning mode. Typical bleaching times ranged from 100 to 500 ms. Bandwidth settings of 20, 30 and 40 nm were also used for testing the most efficient conditions for spectral unmixing.

Spectral compositional of fluorescence emission from ECFP-EYFP and ECFP-Venus

The equations listed below were employed to process and analyse fluorescence emission intensities sampled at different wavelengths (λmax). The program EXCEL (Microsoft Corp.) was used to perform calculations. To obtain background-subtracted fluorescence intensity data of FPs (iFP), intensities measured for BG were subtracted from data of FP-expressing cells (Eq. 1). Here, compensation for different cell numbers was performed using emission intensities at 612 nm (for SP2 spectra alike).

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\[ \text{SP}_1^{\text{FP}}(\lambda_{\text{EM}}) = \text{SP}_1^{\text{FP}}(\lambda_{\text{EM}}) \times \left(1 - \frac{\text{SP}_1^{\text{FP}}(612\text{nm})}{\text{SP}_1^{\text{BG}}(612\text{nm})}\right) \]  

In SP1 of ECFP-Venus, emission from acceptor fluorophores caused by direct excitation (DE) at \( \lambda_{\text{EX}} = 430 \text{ nm} \) was estimated using spectral characteristics of Venus as a reference. Here, intensities measured at \( \lambda_{\text{EM}} = 527 \text{ nm} \) in SP2 provide the scaling factor used to compensate for signal intensity differences (Eq. 2).

\[ \frac{\text{SP}_1^{\text{ECFPVENUS}}(\lambda_{\text{EM}})}{\text{SP}_1^{\text{Venus}}(\lambda_{\text{EM}})} = \frac{\text{SP}_1^{\text{ECFPVENUS}}(527\text{nm})}{\text{SP}_1^{\text{Venus}}(527\text{nm})} \]  

The contribution of ECFP emission (EE) to SP1 of ECFP-Venus was estimated using SP1 of ECFP as a reference. Intensities measured at the emission maximum of ECFP (\( \lambda_{\text{EM}} = 476 \text{ nm} \)) provide the scaling factor used to compensate for signal intensity differences (Eq. 3).

\[ \frac{\text{SP}_1^{\text{ECFPVENUS}}(\lambda_{\text{EM}})}{\text{SP}_1^{\text{ECFP}}(\lambda_{\text{EM}})} = \frac{\text{SP}_1^{\text{ECFPVENUS}}(476\text{nm})}{\text{SP}_1^{\text{ECFP}}(476\text{nm})} \]  

FRET: the contribution of sensitized emission (SE) to SP1 of ECFP-Venus was determined according to Eq. (4).

\[ \frac{\text{SP}_1^{\text{ECFPVENUS}}(\lambda)}{\text{SP}_1^{\text{ECFPVENUS}}(\lambda_{\text{EM}})} = \frac{\text{SP}_1^{\text{ECFPVENUS}}(\lambda_{\text{EM}})}{\text{SP}_1^{\text{ECFPVENUS}}(\lambda_{\text{EM}}) - \text{SP}_1^{\text{ECFPVENUS}}(\lambda_{\text{EM}})} \]  

Data obtained of ECFP-EYFP were analysed using the same equations. Here, spectra of EYFP instead of Venus were used as a reference.