ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/230005741

Confocal microscopy of single molecules of the green fluorescent protein

ARTICLE *in* BIOIMAGING · MARCH 1998

DOI: 10.1002/1361-6374(199803)6:1<54::AID-BIO7>3.0.CO;2-8

CITATIONS
34

READS
50

7 AUTHORS, INCLUDING:

Jens Wiehler
Eurofins, Germany
19 PUBLICATIONS **424** CITATIONS
SEE PROFILE

Christoph Bräuchle
Ludwig-Maximilians-University of Munich
388 PUBLICATIONS **8,571** CITATIONS
SEE PROFILE

All in-text references underlined in blue are linked to publications on ResearchGate, letting you access and read them immediately.

Available from: Christoph Bräuchle
Retrieved on: 26 January 2016

Bioimaging **6** (1998) 54–61. Printed in the UK PII: S0966-9051(98)90242-8

Confocal microscopy of single molecules of the green fluorescent protein

G Jung†, J Wiehler‡, W Göhde§, J Tittel†, Th Basché‖, B Steipe‡ and C Bräuchle†¶

† Institut für Physikalische Chemie, Universität München, Sophienstrasse 11, 80333 München, Germany
‡ Genzentrum der Universität München, Feodor-Lynen-Strasse 25, 81377 München, Germany
§ Physikalisches Institut, Universität Münster, Wilhelm-Klemm-Strasse 10, 48149 Münster, Germany
‖ Institut für Physikalische Chemie, Universität Mainz, Jakob-Welder-Weg 25, 55099 Mainz, Germany

Submitted 19 November 1997, accepted 23 December 1997

Abstract. Single molecule detection has been extended into life sciences by use of strongly fluorescent labels. The green fluorescent protein (GFP) as a self-fluorescent biomolecule has attracted considerable attention. Here, single molecules of the GFP-mutant Glu222Gln are immobilized in a polyvinylalcohol matrix and detected by confocal fluorescence microscopy. Although this mutant stabilizes one of both conformers of the wild-type GFP, the investigation of its fluorescence dynamics reveals strong signal fluctuations. This fluorescence behaviour is—at least partly—caused by reversible photochemical changes of the protein framework, that can relax into the fluorescent state on different timescales. Thus, this protein appears particularly appropriate for studying the microheterogeneity of the macromolecule GFP on a single molecule level.

Keywords: GFP, single molecule detection, confocal fluorescence microscopy, photochemistry, blinking

1. Introduction

Single molecule spectroscopy has attracted considerable attention in the past few years. While in conventional experiments only ensemble averaged values can be measured, single molecule spectroscopy allows one to investigate the individual properties of a single molecule. In this way quantum optical behaviour can be observed, for example photobleaching of a single molecule results in a sudden turn-off of the fluorescence (one-step process) whereas for an ensemble of molecules an exponential decay would be obtained. Furthermore, in heterogeneous systems, inhomogeneities can be uncovered which are present due to the individual local environment of each molecule. In ensemble measurements only inhomogeneous broadening would be observed. Tremendous progress has been made in the different schemes for detection, imaging and spectroscopy of single molecules (for reviews see [1, 2]). Apart from the spectral selection of single molecules at cryogenic temperatures (for reviews see [3, 4]), microscopic detection schemes have been developed for spatial separation (for reviews see [5, 6]) allowing work at room temperature. Observation of single molecules in solution [7, 8], at interfaces and in or on thin polymeric films [9, 10] with farfield, nearfield and confocal microscopy as well as imaging of moving molecules with a CCD camera [11, 12] have been demonstrated. In the meantime the application of single molecule detection was extended into the field of biology using biomolecules labelled with strong fluorescent dyes [11, 13]. A new approach in this field focuses on (self-) fluorescent proteins. These proteins normally need cofactors for fluorescence like NADH or flavine mononucleotide. The novelty of the green fluorescent protein (GFP) from the pacific

¶ Author to whom correspondence should be addressed.

0966-9051/98/010054+08$19.50 © 1998 IOP Publishing Ltd

jellyfish *Aequorea victoria* is that its fluorescence does not require any cofactors as proven by expression in pro- and eukaryotic organisms [14]. Its structural analysis reveals the autocatalytical formation of the chromophore by cyclization of the amino acids 65–67 [15–17]. Since the discovery of its outstanding properties, GFP has become an important marker of gene expression and localization of the encoded proteins in cells. Manipulation of the amino acid sequence has resulted in different fluorescing proteins like blue, cyan or yellow–green GFP [18]. In a recent development, a fluorescent *in vivo* Ca^{2+}-indicator was realized by Förster resonance energy transfer between different mutants [19]. The photophysics of the large Stokes shift in the fluorescence of GFP was investigated by ultrafast dynamical studies [20, 21]. Recently, single GFP molecules were immobilized in a gel matrix and imaged by the total-internal-reflection method [22].

In view of the manifold use of GFP in molecular biology, it is important to understand its photophysics. The investigation of its spectral features is the basis for further extensive use in labelling biomolecules and detection on a single molecule level. Here we present the imaging and spectroscopy of single molecules of the GFP-mutant Glu222Gln by scanning confocal microscopy and the investigation of its fluorescence dynamics; the results are discussed with respect to the suitability as a dye for single molecule applications and as an object for studies concerning protein dynamics.

2. Experiment

2.1. Material and sample preparation

GFP used in this study was based on the pGFP gene [14] containing the Gln80Arg mutation. To prepare the gene for expression and purification, its NcoI restriction site was eliminated by site directed mutagenesis [23]. The resulting gene was amplified by PCR, using primers which introduced a novel NcoI site overlapping with the initiation ATG at the 5′-end and a Bst EII site at the 5′-end. Due to required sequence changes, the N-terminal Ser_2 has been changed to Gly and another Gly has been appended to the C-terminus. The PCR fragments were digested, purified and cloned into plasmid pPHCy1 [24] resulting in the expression plasmid pt7GFPav. Expression is under control of the T7 promoter and one-step purification of the expressed protein is possible via a C-terminal His_6 tag. The mutant Glu222Gln was constructed by site directed mutagenesis [23] and the mutation was confirmed by DNA sequencing. The proteins were expressed in *E. coli* BL21 DE3 at 25 °C. Expression was induced with 0.5 mM IPTG and cells harvested after 4 h. After sonification, the total cytoplasmic protein was applied to a Ni-NTA agarose column (Quiagen). Columns were washed (40 mM imidazole, 300 mM NaCl, 50 mM Na_2HPO_4) and eluted by increasing the imidazole to 0.5 M. SDS-page indicated a purity greater than 99%. Final yields were 3–9 mg of purified protein per litre of culture.

A 10^{-4} M solution of the GFP mutant in buffer was diluted to 5×10^{-8} M in a polyvinylalcohol (pva) (Fluka, MW 49 000) containing solution ($\approx$ 20 mg ml^{-1}). Since we use buffer solutions and a concentration below 10^{-4} M, we can exclude dimerization or agglomerations of the cytosolic protein. Spin coating of this solution yields polymer films (thickness 40–70 nm) where single molecules are embedded. This preparation results in a theoretical concentration of 1–2 molecules μm^{-2} and a detection volume below 3×10^{-2} fl, taking the sample thickness into account.

2.2. Set-up

The experimental set-up of our confocal microscope is sketched in figure 1 and will be described briefly. Several μW of the light output of a krypton-ion laser (476 nm) are focused onto a pinhole, reflected by a dichroic mirror (Omega, 475DCLP02) and are then focused on the sample by a microscope objective (Newport, 60×, NA 0.85). Fluorescence of the sample excited by the bluegreen light is collected by the same objective. The fluorescence light passes the dichroic mirror and is separated by a holographic notch filter (Kaiser, Notch-Plus) from residual excitation light. Spatial filtering from background light is achieved by the detection pinhole (diameter 200 μm). For adjustment purposes, a removable mirror allows imaging of the focus on a colour video-CCD. For the generation of images and the registration of time traces, the fluorescence light is deflected on a lens and then refocused. Its detection is carried out by an avalanche photodiode (EG&G, SPCM-200, dark count rate below 4 s^{-1}). The detection efficiency is about 3% for fluorescence photons (500 nm). Without mirrors, light is dispersed by a spectrograph (Jobin Yvon, HR 460) and detected by a liquid-nitrogen cooled CCD (Princeton Instruments, ST 138), the spectral resolution being 0.8 nm. The sample is prepared on a cover slip and then mounted on a piezo scanner for a two-dimensional scanning. The scan area is 8×8 μm^2, each axis being divided into 128 pixels. A more detailed description of the apparatus is given in [25]. For a better visualization of single molecules, we apply a uniform filter with a 3×3 kernel. This operation reduces the noise on the fluorescence light, however, with the drawback of a reduced resolution. The lateral resolution is then about 400 nm. Addressing single bright spots in the fluorescence image, time traces and fluorescence spectra from these spots can be taken. All experiments are performed at ambient temperature under a helium atmosphere.

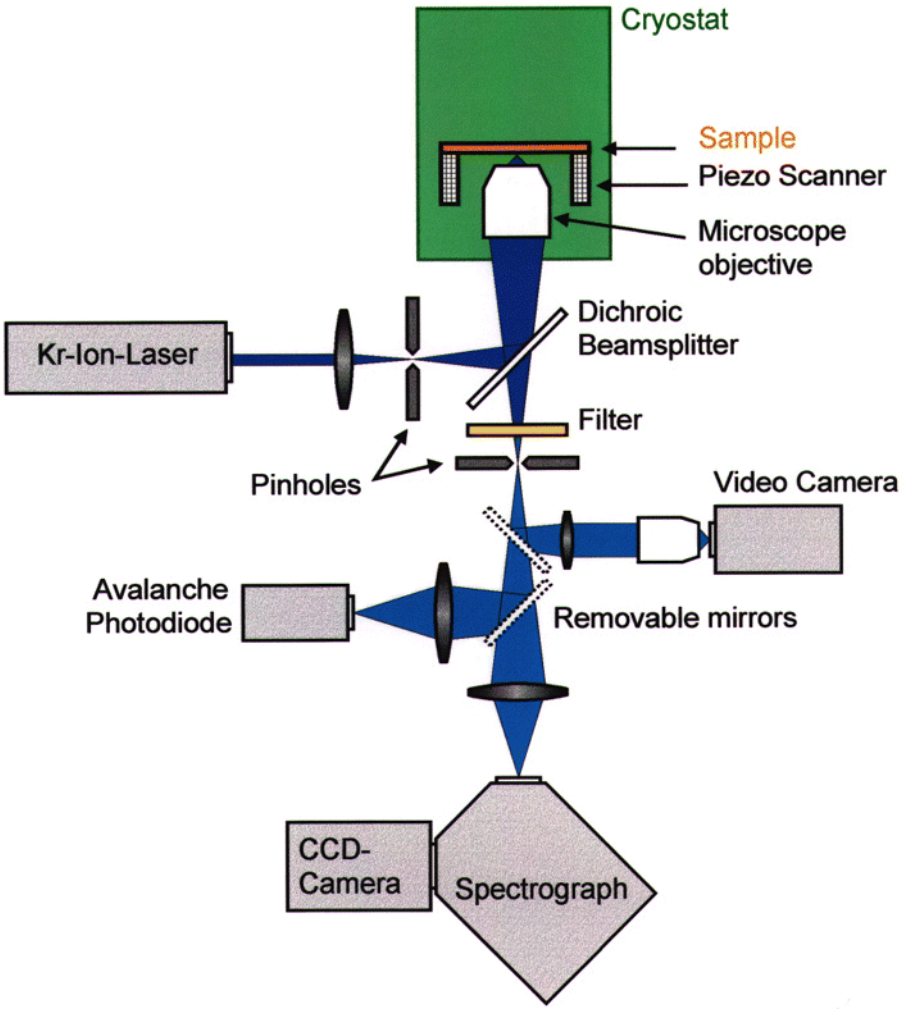


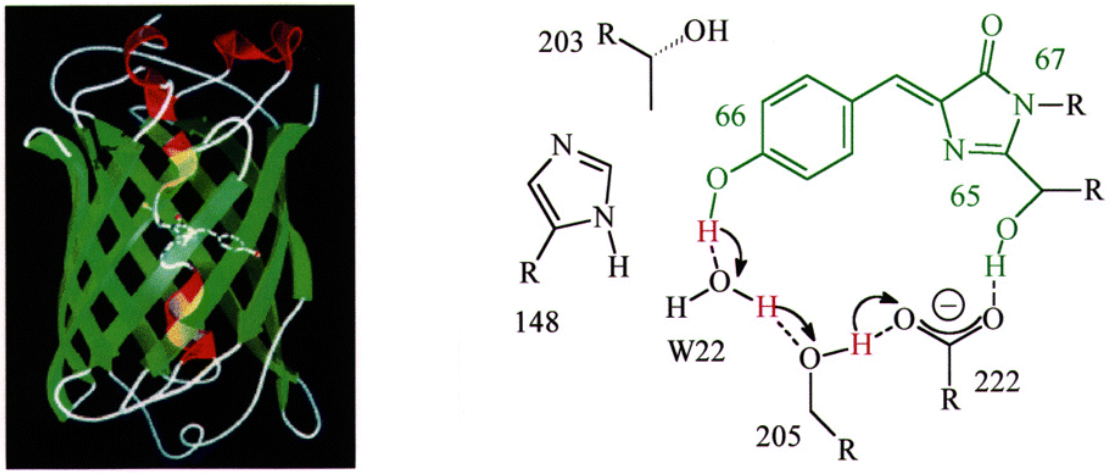
Figure 1. The experimental set-up of the confocal microscope (see text for details).

3. Results and discussion

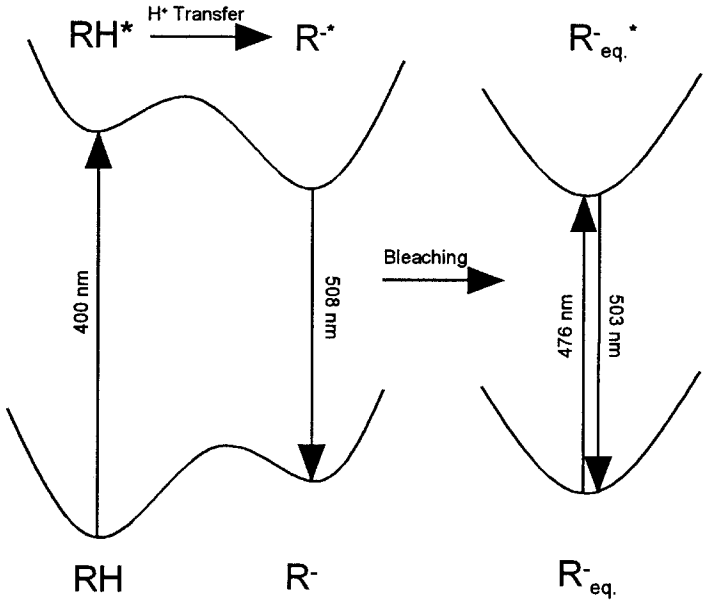
3.1. Properties of GFP

The chromophore of the wild type (wt)-GFP is a p-hydroxybenzylidene-imidazolinone which consists of the three amino acids Gly65, Tyr66 and Ser67. The structure and the inner part of the protein is shown in figure 2(a) [17]. The first step in the chromophore formation is the cyclization of the imidazolinone ring; then, in an oxidation process the conjugated π-system is completed [26]. The photophysics can be described in the scheme of figure 2(b) [21]. The protonated chromophore (RH) of the wt-GFP absorbs light with a maximum near 400 nm ($\varepsilon \approx 25\,000\ M^{-1}\ cm^{-1}$). In the excited state, a proton is transferred to acceptor groups in an H-bonding framework forming a R^{-*}-state which shows fluorescence $R^{-*} \rightarrow R^{-}$ at 510 nm. Besides the R^{-}-anion, there exists a second stable conformer, R^{-}_{eq}, which is thought to be in a more equilibrated ground state. Its excitation (maximum near 476 nm, $\varepsilon \approx 7000\ M^{-1}\ cm^{-1}$) leads to a fluorescence at 503 nm. The ratio of both conformers can be influenced by the solvent [21]. Conversion of RH into R^{-}_{eq} occurs by longtime illumination with violet light. For this process, rearrangements in the amino acid backbone must take place [17]. In a simplified picture, the dynamics can be reduced to the competition between the acidity of the phenolic OH-group of Tyr66 and glutamic acid Glu222. Depending on conformational changes, the protonated or the deprotonated state of the chromophore is favoured. Thus, relaxation into the chemical equilibrium between both forms is slow. Mutations in the amino acid sequence of the wt-GFP can stabilize one of both forms.

The mutant Glu222Gln, where glutamic acid (Glu) in its anionic form is replaced by glutamine (Gln), interrupts the H-bonding framework. The structural changes should be minor since both amino acids are of comparable size. However, the replacement stabilizes the deprotonated chromophore since the acidity on position 222 is largely reduced. The effect mainly concerns the chemical equilibrium between the conformers which is shifted to the



(a)



(b)

Figure 2. (a) The tertiary structure of GFP with the chromophore in its centre, shielded from the outer environment. The chromophore (p-hydroxybenzylidene-imidazolinone, green line) is shown with some important amino acids in its protonated form (RH). The model and structure are adapted and modified from Brejc *et al* [17]. (b) A simplified energy scheme of wt-GFP (adapted from [21]) showing the excited state, proton transfer and the bleaching into an equilibrated state of the anion [20].

deprotonated form, so that no protonated chromophore is observed in bulk spectra. This does not affect the electronic structure of the deprotonated chromophore. Therefore, the only excitation maximum of this mutant is at 478 nm, and it leads to fluorescence with maximum at 506 nm and a fluorescence yield comparable to that of wt-GFP. In summary, from the mutant Glu222Gln used in our experiments, we expect that the photocycle with excitation at 476 nm and emission at 506 nm of the equilibrated anion R^-_{eq} (corresponding to the right part in figure 2(b)) is stabilized and fewer side reactions leading to, for example, the proton transfer cycle (left part in figure 2(b)) are possible. This expectation can be critically investigated with the highly sensitive single molecule spectroscopy.

3.2. Single molecule detection

Figure 3(a) shows a typical fluorescence image of the previously described samples. It is scanned from the lower left corner to the lower right corner in 128 steps and then linewise from the bottom to the top in 128 lines. Each pixel represents a 30 ms time period and contains the number of detected fluorescence photons. The image shows several fluorescence spots (maximum about 26 counts in 30 ms at an illumination intensity of 0.5 kW cm^{-2}). A Gaussian profile can be fitted to the bright spots with a full

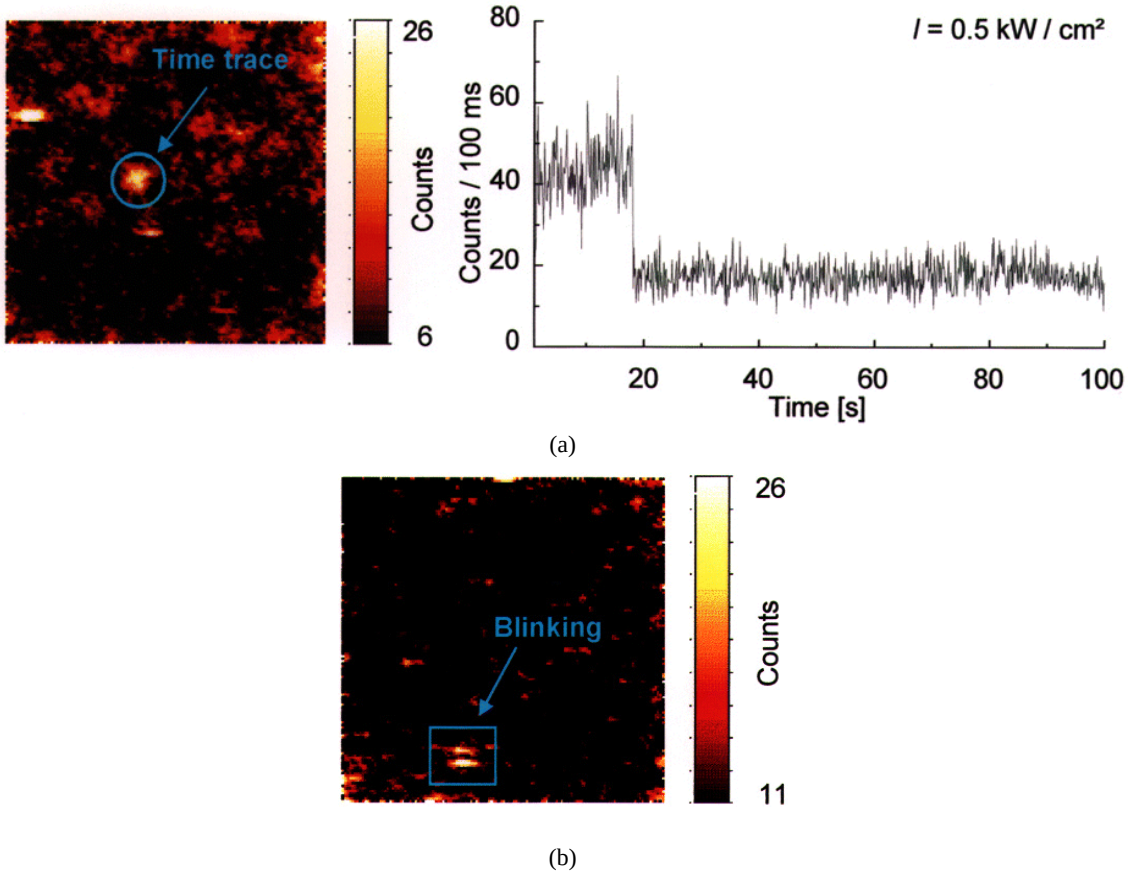


Figure 3. (a) The fluorescence image (8×8 μm^2, 128×128 pixel) of a low covered GFP sample. From the spot indicated by the circle the fluorescence time trace is shown with a 100 ms time resolution. (b) A so-called blinking molecule marked by the rectangular area. During the scan (linewise from bottom to top), the fluorescence stops and re-occurs after several seconds. This effect is seen by a dark stripe in the spot.

width half maximum (FWHM) of 400 ($\pm$50) nm, indicating a sub-resolution fluorescence point. From the excitation intensity, we can estimate an upper limit of 3000 excitation cycles per 30 ms. With a fluorescence quantum yield of about 70% (as in many mutants) and a detection efficiency of about 3%, not more than 60 fluorescence counts are expected. This rough estimation does not include off-times of the molecule and the random orientation with respect to the polarization of the excitation light. These processes reduce the observable fluorescence counts. Single spots which can be detected show maximally 30 counts in 10 ms at a power of 1.5 kW cm^{-2} or in 30 ms at a power of 0.5 kW cm^{-2} (background 6–8 counts), which is in good agreement to what is expected for the detection of single GFP molecules. Furthermore, strong evidence for single molecule detection is also given by the time traces (figure 3(a)): there we have focused on a single fluorescence spot and followed the fluorescence intensity in 100 ms intervals (excitation intensity 0.5 kW cm^{-2}). This time trace shows a sudden disappearance of fluorescence down to the background light level. This 'one-step' behaviour is typical for single molecules. This finding is confirmed by the phenomenon of 'blinking' (figure 3(b)): during the linewise scan a single molecule stops emitting fluorescence. The following dark time (off-time), which can be seen as a dark stripe in the spot of the image, is followed by re-occurrence of the fluorescence (on-time).

In figure 4 two images and spectra of two samples are shown which differ in the coverage density of GFP molecules (factor 100). This difference is evident by the brightness of the images. In both pictures fluorescence spectra are taken. The lower spectrum shows the emission spectrum of one or not more than two molecules and resembles the high coverage spectrum above which is a bulk fluorescence spectrum of GFP. Thus, this proves that the fluorescence spots are caused by GFP molecules. Summarizing all described observations, we can clearly conclude that we investigate single GFP molecules.

3.3. Dynamic behaviour

The temporal evolution of the emission, called time traces, also exhibits strong fluorescence dynamics below a 100 ms resolution as shown before (figure 3(a)). The strong signal fluctuations can be explained by looking on a shorter timescale (figure 5). From a bright spot, representing a

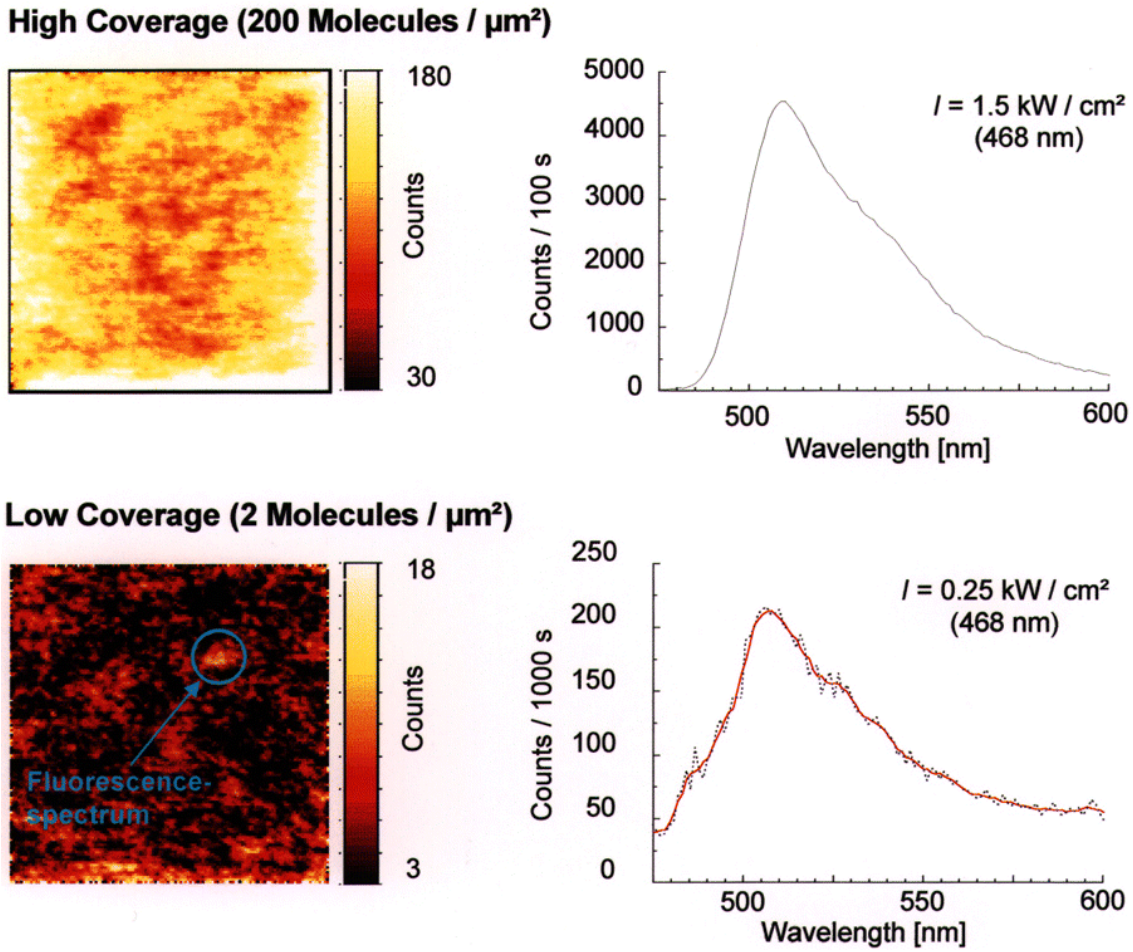


Figure 4. Fluorescence spectra from a densely covered sample (about 200 molecules μm^{-2}) (top) and a sparsely covered sample (about 1–2 molecules μm^{-2}) (bottom).

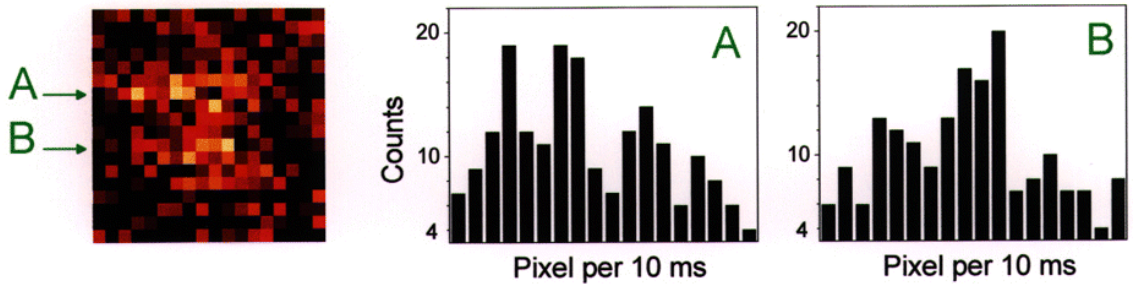


Figure 5. The fluorescence image of a single molecule on the left (10 ms per pixel, raw data). Profiles parallel to the horizontal axes (two examples are marked by an arrow) represent the temporal evolution (here) on a 10 ms timescale.

single molecule, horizontal profiles are extracted in figure 5, which include the temporal evolution during a scan (the two profile lines are marked by arrows). Each pixel represents a 10 ms time period and the profile line is thus similar to a time trace. Although the histograms in figure 5 are not corrected for a Bessel-like excitation profile, on–off dynamics on a several ms timescale are obvious for individual molecules. The results suggest fluorescence dynamics on a 10 ms timescale or faster. Fluorescence correlation spectroscopy on (numerous) single molecules of our mutant with a higher time resolution are under way and will reveal more details about the fast on–off dynamics. Preliminary results of other groups [27] on other mutants also show such fast dynamics. So far we cannot identify the nature of such short time dark states. In principle, these metastable states can be long-lived triplet states or can be caused by fast spectral diffusion. In the case of GFP, where the chromophore is completely surrounded by the can-like structure of the protein, spectral diffusion can have its origin in conformational changes of the macromolecule GFP or in

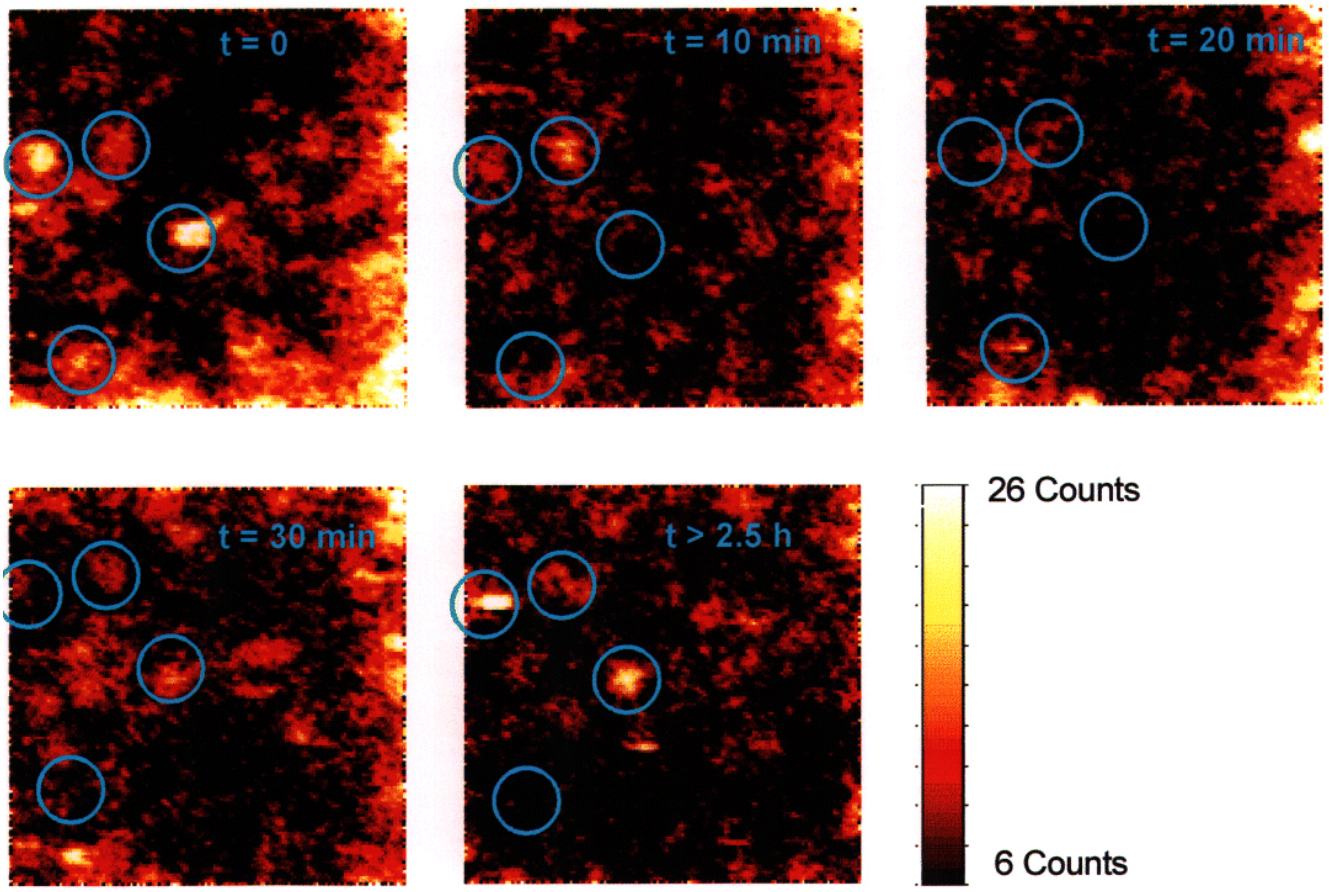


Figure 6. A sequence of fluorescence images ($8 \times 8\ \mu m^2$), taken at different times from the same sample area. Molecules have been marked by circles to facilitate identification from scan to scan.

changes of the outer solvent respectively polymer matrix environment. However, due to the distance between the solvent shell of the protein and the chromophore, their interaction should be small (see figure 2(a)).

Besides the fast fluorescence dynamics, the mutant Glu222Gln also shows a reversible on–off behaviour on a much longer timescale. Figure 6 shows the fluorescence images taken at $t = 0$, $t = 10$ min, $t = 20$ min, $t = 30$ min and $t > 150$ min from the same sample area. It is known from calibration experiments that both the short and the long time drift of our scanner lead to a spatial shift of less than 10% for each axis. The illumination intensity of a molecule during the scanning corresponds to an excitation of about 450 000 photocycles per image. It is highly remarkable that single molecules indicated by circles in figure 6 disappear after the first image into a dark state and reappear after 150 min. From this we have to conclude that the relaxation from such a dark state back to a fluorescent state (excitation wavelength at 476 nm) can take a very long time. This long time behaviour may suggest that larger rearrangements of the protein framework take place. It is known [17] that such rearrangements in the protein backbone (e.g. T203) contribute to changes in the H-bonding framework. It may be that in some cases the protonated form RH of the chromophore is produced (which would be a dark state for the excitation wavelength ($\lambda = 476$ nm). Such a state will relax on a very long time scale into the equilibrium state [20], which for our mutant is R^-_{eq}. However, we cannot exclude other mechanisms like, for example, spectral diffusion—as discussed before—which can also occur on a long time scale.

In further experiments we will try to shed more light onto the identification of long and short time dark states. For the mutant Glu222Gln, which should show a high stability of the $R^-_{eq} \Leftrightarrow R^{-*}_{eq}$ photocycle, however, one has to conclude that reversible side reactions to other states with a large variety of dark times take place. This reflects the high degree of microheterogeneity which has to be taken into account for such a (large) macromolecule like GFP. Single molecule spectroscopy is sensitive enough to show this effect clearly.

4. Conclusions

In this paper we have presented the detection of single fluorescent molecules of the GFP-mutant Glu222Gln. This mutation stabilizes the deprotonated chromophore

of the wt-GFP. The molecules are embedded in a pva-matrix which seems to be an appropriate medium for the detection of single GFP molecules. Blinking as a one-step behaviour in the fluorescence as well as the fluorescence spectrum prove that single GFP molecules have been observed. Although the mutant used here was improved with respect to the interconversion between the protonated and deprotonated conformers in the wild-type, fluctuations in the fluorescence time trace on different time scales (from ms to s to h) may be—at least partly—attributed to reversible interconversions between different conformers of GFP. In this way the highly sensitive single molecule detection is able to show the microheterogeneity of the macromolecule GFP.

Acknowledgments

JW and BS thank Heike Bruhn and Christine Samanns for laboratory work. This work was supported by the Fond Chemischer Industrie and the DFG.

References

[1] Basché T, Moerner W E, Orrit M and Wild U P (eds) 1997 *Single Molecule Optical Detection, Imaging and Spectroscopy* (Munich: Chemie)

[2] Barbara P F and Moerner W E (eds) 1996 Single molecules and atoms *Acc. Chem. Res.* **29** 563–613

[3] Moerner W E 1994 Examining nanoenvironments in solids on the scale of a single, isolated impurity molecule *Science* **265** 46–52

[4] Orrit M, Bernard J and Personov R I 1993 High-resolution spectroscopy of organic molecules in solids: from fluorescence line narrowing and hole burning to single molecule spectroscopy *J. Phys. Chem.* **97** 10 256–68

[5] Xie S X 1996 Single molecule spectroscopy and dynamics at room temperature *Acc. Chem. Res.* **29** 598–606

[6] Nie S, Chiu D T and Zare R N 1994 Probing individual molecules with confocal fluorescence microscopy *Science* **266** 1018–21

[7] Goodwin P M, Ambrose W P and Keller R A 1996 Single-molecule detection in liquids by laser-induced fluorescence *Acc. Chem. Res.* **29** 607–13

[8] Eigen M and Rigler R 1994 Sorting single molecules: applications to diagnostics and evolutionary biotechnology *Proc. Natl Acad. Sci., USA* **91** 5740–7

[9] Macklin J J, Trautman J K, Harris T D and Brus L E 1996 Imaging and time-resolved spectroscopy at an interface *Science* **272** 255–8

[10] Betzig E and Chichester R J 1993 Single molecules observed by near-field scanning optical microscopy *Science* **262** 1422–5

[11] Schmidt T, Schütz G J, Baumgartner W, Gruber H J and Schindler H 1996 Imaging of single molecule diffusion *Proc. Natl Acad. Sci., USA* **93** 2926–9

[12] Dickson R M, Norris D J, Tzeng Y L and Moerner W E 1996 Three dimensional imaging of single molecules in pores of poly(acrylamide) gels *Science* **274** 966–9

[13] Funatsu T, Harada Y, Tokunaga M, Saito K and Yanagida T 1995 Imaging of single fluorescent molecules and individual ATP turnover by single myosin molecules in aqueous solution *Nature* **374** 555–9

[14] Chalfie M, Tu Y, Euskirchen G, Ward W W and Prasher D C 1994 Green fluorescent molecule as a marker for gene expression *Science* **263** 802–4

[15] Ormö M, Cubitt A B, Kallio K, Gross L A, Tsien R Y and Remington S J 1996 Crystal structure of the *Aequorea victoria* green fluorescent protein *Science* **273** 1392–5

[16] Yang F, Moss L G and Philips G N 1996 The molecular structure of green fluorescent protein *Nature Biotechnol.* **14** 1246–51

[17] Brejc K, Sixma T K, Kitts P A, Kain S R, Tsien R Y, Ormö M and Remington S J 1997 Structural basis for dual excitation and photoisomerization of the *Aequorea victoria* green fluorescent protein *Proc. Natl Acad. Sci., USA* **94** 2306–11

[18] Cubitt A B, Heim R, Adams S R, Boyd A E, Gross L A and Tsien R Y 1995 Understanding, improving and using green fluorescent proteins *Trends Biochem. Sci.* **20** 448–55

[19] Miyawaki A, Llopis J, Heim R, McCaffery J M, Adams J A, Ikura M and Tsien R Y 1997 Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin *Nature* **388** 882–7

[20] Chattoraj M, King B A, Bublitz G U and Boxer S G 1996 Ultra-fast excited state dynamics in green fluorescent protein: Multiple states and proton transfer *Proc. Natl Acad. Sci., USA* **93** 8362–7

[21] Lossau H *et al* 1996 Time-resolved spectroscopy of wild-type and mutant green fluorescent proteins reveals excited state deprotonation consistent with fluorophore–protein interactions *Chem. Phys.* **213** 1–16

[22] Dickson R, Cubitt A B, Tsien R Y and Moerner W E 1997 On/off blinking and switching behaviour of single green fluorescent protein molecules *Nature* **388** 355–8

[23] Kunkel T A 1985 Rapid and efficient site-specific mutagenesis without phenotypic selection *Proc. Natl Acad. Sci., USA* **82** 488–92

[24] Bruhn H 1997 unpublished results

[25] Goehde W, Tittel J, Basché T, Bräuchle C, Fischer U C and Fuchs H 1997 A low temperature scanning confocal and near-field optical microscope *Rev. Sci. Instrum.* **68** 2466–74

[26] Reid B G and Flynn G C 1997 Chromophore formation in green fluorescent protein *Biochemistry* **36** 6786–91

[27] Rigler R 1997 private communication