Photoactivation turns green fluorescent protein red

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In the few years since its gene was first cloned, the *Aequorea victoria* green fluorescent protein (GFP) has become a powerful tool in cell biology, functioning as a marker for gene expression, protein localization and protein dynamics in living cells [1–3]. GFP variants with improved fluorescence intensity and altered spectral characteristics have been identified, but additional GFP variants are still desirable for multiple labeling experiments, protein interaction studies and improved visibility in some organisms [4]. In particular, long-wavelength (red) fluorescence has remained elusive. Here we describe a red-emitting, green-absorbing fluorescent state of GFP that is generated by photoactivation with blue light. GFP can be switched to its red-emitting state easily with a laser or fluorescence microscope lamp under conditions of low oxygen concentration. This previously unnoticed ability enables regional, non-invasive marking of proteins *in vivo*. In particular, we report here the use of GFP photoactivation to make the first direct measurements of protein diffusion in the cytoplasm of living bacteria.

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Results and discussion

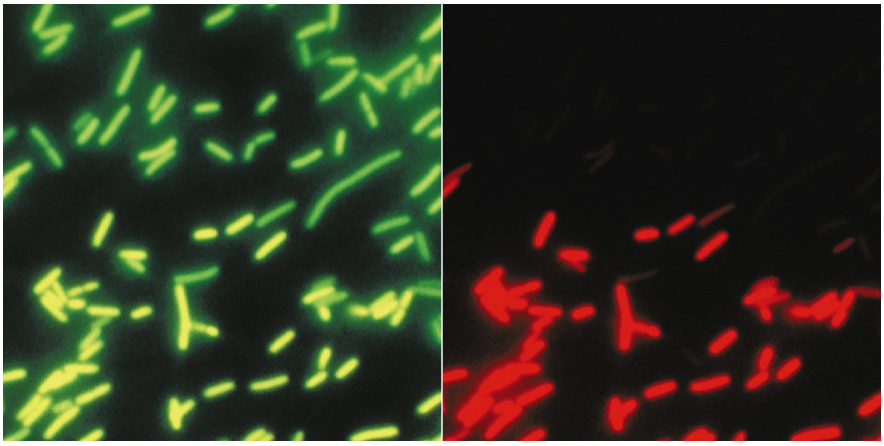
Cells expressing commonly used 'enhanced-brightness' GFP variants absorb blue light (480–500 nm) and emit green light (507–511 nm) [4–6]. The exact positions of the absorption peaks depend on the specific GFP variant under consideration, but their spectra are generally similar; in particular, none of them displays significant red fluorescent emission. Under some conditions, however, we have found that exposure to exciting blue light generates a change in the GFP fluorescence spectrum, so that GFP begins to absorb green light (around 525 nm) and emit green, yellow and red light (emission maxima around 560, 590 and 600 nm; see below). This new GFP fluorescence shows up brightly inside living cells as well as in samples of purified protein, and is easily distinguishable from the usual green GFP fluorescence (Figure 1).

Photoactivation of GFP occurs in a low oxygen environment. Using a simple microscope assay (see Materials and methods), we observed that purified GFP could be photoactivated when prepared in a nitrogen environment, or when mixed with an O_2-scavenging system consisting of glucose oxidase, catalase and β-D-glucose. GFP-expressing *Escherichia coli* were also found to be photoactivatable when O_2 scavengers were added to their media. In addition, thin layers of bacteria sealed in microscope slides became photoactivatable within ~25 minutes, presumably because of their own oxygen consumption. The apparent requirement for low oxygen levels may explain why this effect was not observed earlier. The facilitation of GFP photoactivation by low oxygen levels contrasts with the requirement for oxygen during the final stage of GFP chromophore formation [6]. Furthermore, the chromophore in folded GFP appears to be largely inaccessible to oxygen [7–9]. Oxygen's diverse effects on GFP clearly invite closer scrutiny.

We have observed the photoactivation in bacteria of all the GFP variants that we have tested. These include wild-type GFP; the enhanced-brightness mutants (GFPmut1, 2 and 3) described by Cormack *et al.* [5]; the S65T and I167T mutants obtained by Tsien and colleagues [6,10]; and a mutant (GFPuv, Clontech) isolated by Crameri *et al.* [11]. Furthermore, we have been able to photoactivate GFPmut2, both while expressed in *S. cerevisiae* yeast cells (data not shown) and as purified protein. This shows that the effect is not specifically related to expression of GFP by bacteria. The photoactivated form of GFP is stable for at least 24 hours, both in cells maintained in minimal media and as purified protein (data not shown).

To characterize the photoactivated GFP state further, we obtained fluorescence spectra before and after photoactivation. Figure 2a shows the results for pure protein. There is negligible fluorescent emission before photoactivation, but afterwards peaks centered at 600 and 590 nm, as well as a shoulder at 560 nm, have developed. Also shown in Figure 2a are excitation spectra before and after photoactivation. The new excitation feature at 525 nm that arises from photoactivation can be seen by subtracting the excitation spectrum before photoactivation from the excitation spectrum after photoactivation, as shown. Similar,

Figure 1



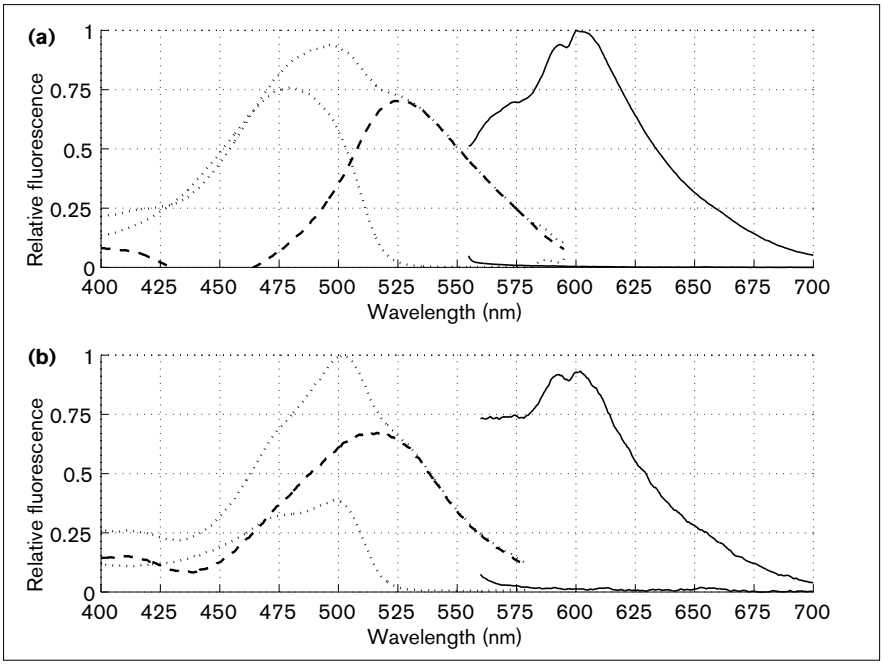
Photoactivation of GFP *in vivo*. GFP-labelled *E. coli* cells appear as viewed through fluorescein (left) and rhodamine (right) filter sets. GFP in cells on the lower half of the images has been photoactivated by exposure to 475–495 nm light. Photoactivated cells appear yellow through the fluorescein filters, because they emit continuously from green to red with local maxima at 510, 560, 590 and 600 nm.

but not identical, spectra are observed for suspensions of GFP-expressing bacteria (Figure 2b). In particular, the emission maxima at 590 and 600 nm are observed after, but not before, photoactivation.

A clue to the structural origin of the state may lie in the slow rate at which it becomes active. We exposed GFP in individual bacterial cells to brief (~30 ms) pulses of 488 nm light, and monitored the total intensity of cellular red fluorescence as a function of time after the end of the photoactivating pulse. The result is a slow brightening with a characteristic (1/e) timescale of about 0.7 sec. From this observation, we propose that photoactivation is a two-step process: 488 nm light stimulates a fast transition to an excited intermediate. This intermediate then decays slowly to the red-emitting GFP state. This second step can evidently proceed in the dark.

The photoactivation effect described here is distinct from the photoisomerization property of wild-type GFP. Excitation of the wild-type protein with UV light was found to enhance its absorbance of blue light and diminish its absorbance of UV light. Many commonly-used GFP variants, such as S65T, lack the UV excitation peak and instead exhibit an enhanced excitation peak around 480 nm. A mechanism for photoisomerization was recently proposed

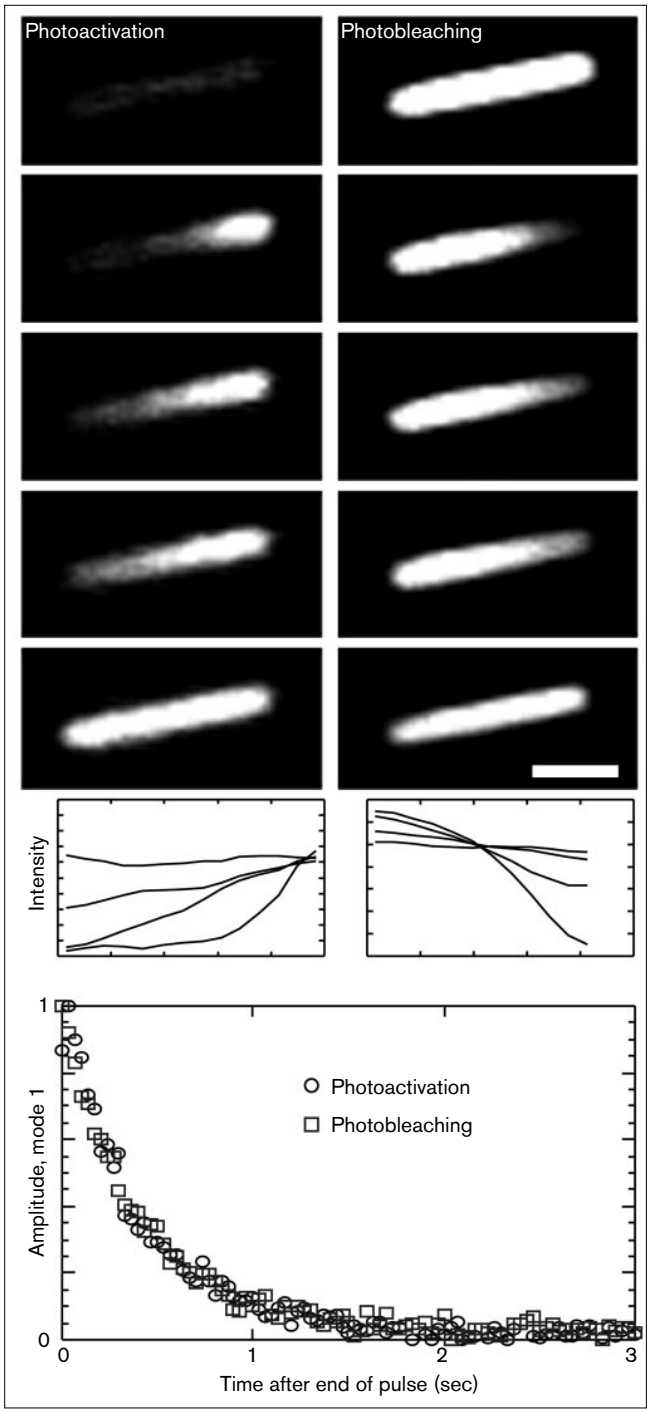
Figure 2



Fluorescence spectra of **(a)** purified GFP, and **(b)** GFP-expressing *E. coli*, before and after photoactivation. Solid lines are emission spectra, obtained with excitation at 546 nm to correspond to the prominent line in the Hg arc lamp spectrum. Dotted lines are excitation spectra (emission at 600 nm). In each pair, the lower and upper curves correspond to before and after photoactivation, respectively. The dashed curves were obtained by subtracting the lower excitation curve from the upper excitation curve. Samples, containing oxygen scavengers, as described in Materials and methods, were photoactivated by directing a 300 mW (1.2 W/cm^2) 488 nm argon laser into the fluorescence cuvette for 20 min after taking the first set of spectra.

based on X-ray crystal structure determinations of wild-type and S65T GFP [12]. The two excitation peaks are thought to correspond to neutral and ionized forms of the GFP chromophore. In the future, a similarly detailed molecular explanation for the photoactivation phenomenon described here should aid in the design of constitutively red mutants and further our understanding of GFP.

In the meantime, the photoactivation characteristic of this new state makes it particularly suitable for measuring protein dynamics *in vivo*, by allowing one to mark molecules in one region and follow their subsequent movements. We have performed such an experiment to measure protein diffusion inside living bacterial cells. The diffusion rate of proteins in bacterial cytoplasm is an important parameter of cellular reaction and signal transduction networks, and a quantity that can be noninvasively measured with this technique. (Details and further extensions of this experiment will be described elsewhere.)



We focused a 488 nm continuous-wave laser through microscope optics to a 0.7 μm spot at one pole of a cell. The power density at the spot was approximately 300 kW/cm^2. With a brief (30 msec) pulse of laser light, we photoactivated the GFP within this region. We observed the red component of the GFP as it diffused throughout the cell (Figure 3, first column). We then switched filters to observe the green fluorescence. A second, longer, laser pulse (0.4 sec) was used to photobleach GFP irreversibly in the same region, after which the fluorescence returned to a uniform distribution (Figure 3, second column). We determined the diffusion coefficient by calculating the first Fourier coefficients from each image and measuring their exponential decay over time.

These measurements show that GFP diffusion in the cytoplasm of *E. coli* strain DH5α is twelve times slower than in water (where D = 87 μm^2/sec) and almost four times slower than the value reported for eukaryotic cytoplasm (D = 27 μm^2/sec in CHO cells) [13]. Specifically, from photoactivation experiments, we obtained an average diffusion constant $D_{act} = 6.7 \pm 1.6$ μm^2/sec, while photobleaching gave us $D_{bleach} = 6.0 \pm 1.0$ μm^2/sec ($n = 19$ cells). The average ratio of D_{act} to D_{bleach} on the same cell was 1.1 ± 0.1 (Figure 3, bottom graph). It is possible to photodamage cells significantly by photobleaching GFP with high-intensity illumination. The photoactivation method has the advantage over photobleaching of requiring significantly

Figure 3

Measurement of GFP diffusion in *E. coli* by photoactivation of red fluorescence (left), and photobleaching of green fluorescence (right). Each column shows snapshots of the cell before (first image) and after (next four images) a laser blast. Images are shown at −0.08, 0.08, 0.35, 0.62, and 4.7 sec, and −0.42, 0.05, 0.18, 0.32, and 4.3 sec, from the end of the laser pulse for photoactivation and photobleaching, respectively. Laser pulse lengths were 30 msec (photoactivation) and 0.4 sec (photobleaching). One-dimensional intensity profiles along the cell at each time step are shown below the images. On the bottom, exponential decay of the first Fourier amplitude is shown for the two techniques. For analysis of photoactivation, the 1–D intensity profiles were normalized independently on each frame by the total (red) fluorescence before Fourier analysis. This was necessary because of the slow photoactivation kinetics of GFP (described in the text), which make the diffusion time along the cell comparable to the photoactivation time. The photoactivation process is not complete at the end of the laser pulse. Rather, it continues even as GFP diffuses through the cell. (Scale bar = 2 μm.)

less energy (a factor of 10 in these experiments). No phototoxicity was observed in our experiments (data not shown).

The GFP state described here may be useful for a wide variety of other experiments in living cells. Photoactivatable GFP can be used in much the same way that caged fluorophores have been used in previous studies, but with certain advantages. Photoactivation of GFP uses less damaging visible, rather than ultraviolet, light, and the gene for GFP can be expressed endogenously and fused to other genes of interest. With its red-emitting state, GFP maintains its reputation as nature's most colorful contribution to cell biology.

Materials and methods

Microscope assay for photoactivation

Microscope assays for photoactivation of GFP were performed using rhodamine (Zeiss, 487915) and GFP/FITC (Chroma, HQ-FITC #41001) or FITC (Zeiss) filter sets on a Zeiss Axiovert microscope equipped with a 100W Hg arc lamp. Photoactivation of one field of view was performed by illuminating at full intensity through the FITC filter set for 2–60 sec. Upon successful photoactivation, the illuminated field of view appears red through the rhodamine filter set, while the area around it (when moved into view with the stage) is dark (Figure 1). In liquid samples, diffusion of GFP will wash out the spot fairly quickly. For this reason, we performed pure protein photoactivation assays in 15% polyacrylamide gels mounted on microscope slides. This method was used to maintain photoactivated spots for long times (24 h).

For photoactivation assays on bacterial cells, coverslips were preincubated with poly-lysine (10%, Sigma) and then rinsed in minimal media (7.6 mM $[NH_4]_2SO_4$, 60 mM K_2HPO_4, 2 mM $MgSO_4$, 30 μM $FeSO_4$, 1 mM EDTA, pH 6.8). *E. coli* cells transformed with plasmid pMGS053, which expresses GFPmut2, were grown and induced as described below, pelletted, resuspended in minimal media, and incubated on treated coverslips for ~20 min. Coverslips were then rinsed with minimal media and placed on slides. Excess fluid was drained with Kimwipes and slides were sealed with wax. Photoactivatability was then checked as described above.

Purified protein spectra

For experiments on purified GFP, we cloned GFPmut2 into pQE8 (Qiagen) by PCR, thus adding an amino-terminal His_6 sequence. This protein was purified on a Ni-NTA column (Qiaexpress, Qiagen) according to the manufacturer's protocol. Mixtures of 260 μg/ml GFP, 0.1 M Tris, pH 7.5, 250 μg/ml glucose oxidase, 30 μg/ml catalase and 4.5 mg/ml β-d-glucose (the last three from Sigma) were covered with a layer of mineral oil (Sigma). High buffer strength was necessary because the oxygen-scavenging reaction generates D-gluconic acid as a by-product. Spectra were acquired on a Perkin-Elmer model MPF-66 fluorescence spectrophotometer (slit size 5 nm) before and after exposing ~500 μl volumes of the protein mixture to a 300 mW, 488 nm laser beam.

E. coli *spectra*

Cell suspensions contained *E. coli* DH5α cells transformed with pMGS053. Cells were grown overnight in LB containing 100 μg/ml ampicillin, then diluted back 1:50 in the same media supplemented with 100 μM IPTG, and grown at 30°C for several hours. They were then resuspended in 0.5 vol. minimal media supplemented with 0.1 M Tris, pH 7.5 and oxygen scavengers at the concentrations given above. Photoactivation parameters (laser power, etc.) were also the same as for the pure protein.

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