Photobleaching GFP reveals protein dynamics inside live cells

Jamie White and Ernst Stelzer

Cell biologists have used photobleaching to investigate the lateral mobility of fluorophores on the cell surface since the 1970s. Fusions of green fluorescent protein (GFP) to specific proteins extend photobleaching techniques to the investigation of protein dynamics within the cell, leading to renewed interest in photobleaching experiments. This article revisits general photobleaching concepts, reviews what can be learned from them and discusses applications illustrating the potential of photobleaching GFP fusion proteins inside living cells.

Photobleaching can be used to reveal the dynamics underlying the steady-state distribution of a fluorophore. A fluorophore within a small region is made non-fluorescent with very high intensity illumination, and exchange between the bleached and unbleached populations of fluorophore is then monitored. In most cases, the exact physical basis for bleaching is not known; the fluorophore is not destroyed but rather irreversibly, photochemically altered so that it no longer fluoresces[1]. There are two variations that yield different types of information: fluorescence recovery after photobleaching (FRAP), also referred to as fluorescence photobleaching recovery (FPR), and fluorescence loss in photobleaching (FLIP). In FRAP, a region is bleached once and then recovery of fluorescence in the bleached zone is monitored. Quantitative FRAP yields information about the relative mobility of the fluorophore: the effective diffusion coefficient (D_{eff}), and the fraction of fluorophore that is mobile[2]. In FLIP, a region is repeatedly bleached, and the loss of fluorescence from outside the bleached region is monitored. FLIP studies show continuity or transport between different populations of fluorophores, such as a green fluorescent protein (GFP) fusion protein localized to two different organelles[3]. In addition to FRAP and FLIP, photobleaching can be used simply to clear an area of unwanted fluorescence, potentially due to overexpression of bright GFP fusion proteins, thus revealing the dynamics of GFP-labelled structures moving into the bleached area[4,5].

Photobleaching GFP

GFP is well suited for photobleaching studies[6,7]. It is a bright, stable, non-toxic fluorophore in live cells and does not bleach significantly under low-intensity imaging conditions. When illuminated at high intensity, GFP bleaches irreversibly without detectably damaging intracellular structures[6–9]. This might be because its compact barrel-like structure buries the fluorophore deep inside the protein[10,11], protecting it from its cellular environment and sheltering the cell from reactive intermediates generated by photobleaching. Different GFP mutants show variations that affect photobleaching characteristics[7]; for quantitative analyses of GFP variants, see Refs 6 and 7. Of those studied, the enhanced GFP variant (EGFP[12]) appears to be one of the most suitable GFP variants for photobleaching studies in tissue-culture cells grown at 37°C[7], although other similar GFP variants might be equally suitable. The S65T variant is similar to EGFP, but folds less well at 37°C[7]. GFP-S65T fluorescence and bleaching properties are resistant to variations in its environment[4], although both EGFP and GFP-S65T fluorescence is reduced at low pH (5.0–6.5)[7,13,14]. GFP bleaches irreversibly under conditions likely to be found within living cells[6–9], although reversible photobleaching has been detected in highly viscous solutions[6]. GFP is much more stable than small fluorescent dyes such as fluorescein isothiocyanate (FITC), allowing long-term observation of the recovery phase, or repeated bleaching and recovery of the same cell[7]. The biophysical properties of GFP make it a useful fluorophore, but the most appealing feature of GFP is that cells can express it themselves, and targeting is intrinsic to the GFP fusion protein. Photobleaching studies have been done in live cells with microinjected fluorescent probes, but this requires invasive and often technically difficult loading procedures. Furthermore, GFP fusion proteins can target to the lumen of intracellular organelles, regions not usually accessible to probes microinjected into the cytoplasm. Published GFP bleaching studies have been performed in tissue-culture cells with both transiently transfected GFP chimeras and stable cell lines. For detailed reviews of GFP photobleaching techniques and applications, see Refs 15 and 16.

Practical matters

Photobleaching experiments require rapid switching between a low-intensity imaging mode and a high-intensity bleach mode during which the bleached region is positioned precisely. Specialized microscopes can be built to achieve this[17], but confocal laser-scanning microscopes (CLSMs) are generally suitable for photobleaching experiments without additional modifications. The relevant feature is laser illumination controlled by acousto-optical devices and positioned with scanners. Acousto-optical devices modulate laser intensity precisely and can switch between high-power bleaching and low-power imaging within milliseconds. Scanners control the position of the laser illumination to select the bleach region accurately. EGFP and GFP-S65T are both excited well at 488 nm with argon-ion

The authors are in the Light Microscopy Group, Cell Biology and Cell Biophysics Programme, European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany.
Emails: jwhite@embl-heidelberg.de
stelzer@embl-heidelberg.de

0962-8924/99/$ – see front matter

PII: S0962-8924(98)01433-0

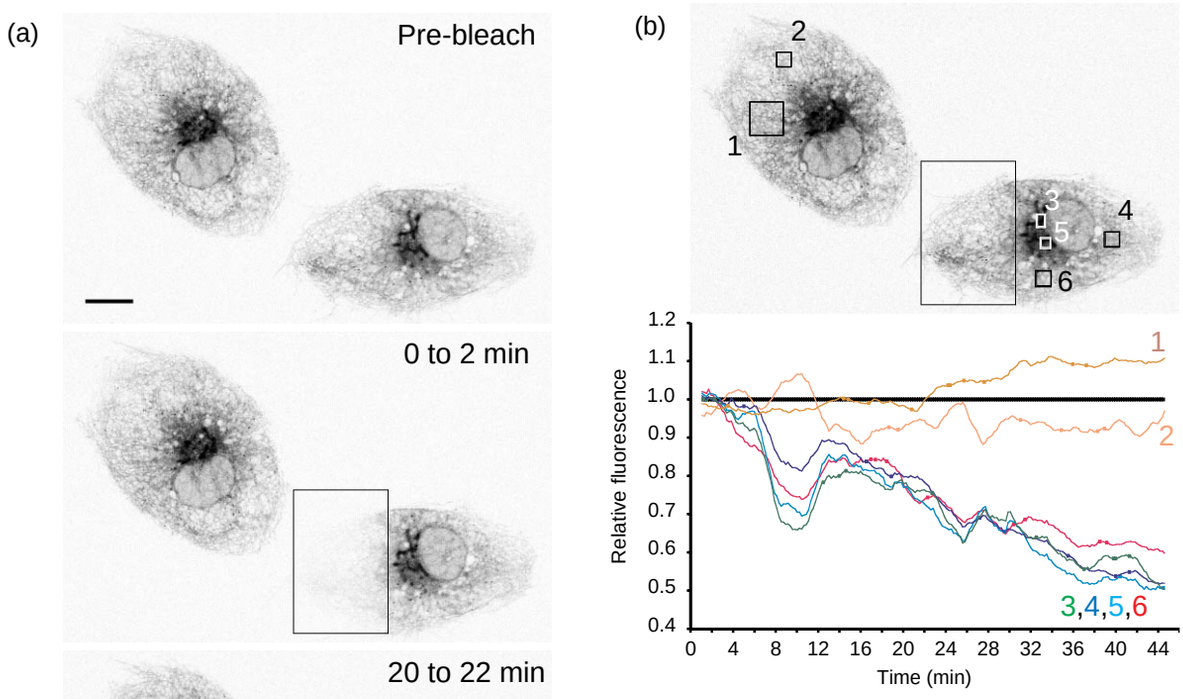

FIGURE 1

Fluorescence loss in photobleaching (FLIP). A green fluorescent protein (GFP) fusion to the KDEL-receptor (KDELR–GFP) was expressed in PtK_2 cells and imaged live on a confocal laser scanning microscope. KDELR–GFP cycles between the endoplasmic reticulum (ER; fine network of fluorescence) and the Golgi apparatus (compact fluorescence adjacent to the nucleus). Repeated bleaching within a region depletes fluorescence from outside the bleached region. (a) The entire field was scanned every 12 s for 10 images, then the boxed region was bleached with 10 scans at full laser intensity. This sequence was repeated 20 times. Shown are averages of 10 imaging scans. The bleached cell gradually became dimmer, while the unbleached control cell remained bright. (b) Over 45 min, the total fluorescence in the bleached cell was reduced to ~40% of its initial value relative to the total fluorescence in the control cell. Similar small regions from the control cell (1, 2) showed minor fluctuations relative to the total fluorescence within the cell. The small regions shown (3–6) became dimmer at similar rates, although two regions (3 and 5) enclosed Golgi elements, and two (4, 6) enclosed ER. This indicates that KDELR–GFP in the Golgi exchanges with KDELR–GFP in the ER. Exchange is not likely to be due to membrane continuity since significant transport can occur over the time required for FLIP. The images are inverted from the usual white-on-black display, so the most intense fluorescence values are darkest. Bar, 10 μm.

lasers; those rated at 10–25 mW have more than sufficient power to bleach GFP.

GFP photobleaching experiments are usually performed on living cells, which requires keeping cells alive on the microscope for long periods under controlled conditions. Laser-scanning microscopy has an undeserved reputation for excessive photodamage of living cells and photobleaching during image acquisition, which if it occurred would complicate collection of fluorescence recovery data[18]. However, the laser intensities used for bleaching GFP do not noticeably perturb live cells. Primary neurons expressing GFP fusions to various trafficking proteins have been repeatedly bleached and observed for periods of hours with a CLSM[5]. Fig. 1 shows a tissue-culture cell that was bleached 22 times over a fairly large area spanning a 45-minute period; it remained morphologically indistinguishable from the control cell, and quantitation of the total fluorescence in the control cell showed that it did not become detectably dimmer over the course of the experiment. Laser scanning microscopy has the additional advantage that the same light used for the fluorescence excitation can also generate simultaneously a DIC/Nomarski image to follow the vitality of cells during photobleaching recovery.

Representative FRAP and FLIP experiments are shown in Figs 1 and 2. Such experiments show how exchange occurs but do not attempt to derive an effective diffusion coefficient (D_{eff}). Quantitation of fluorescence intensity can show relative differences in the mobility of a GFP fusion protein localized to

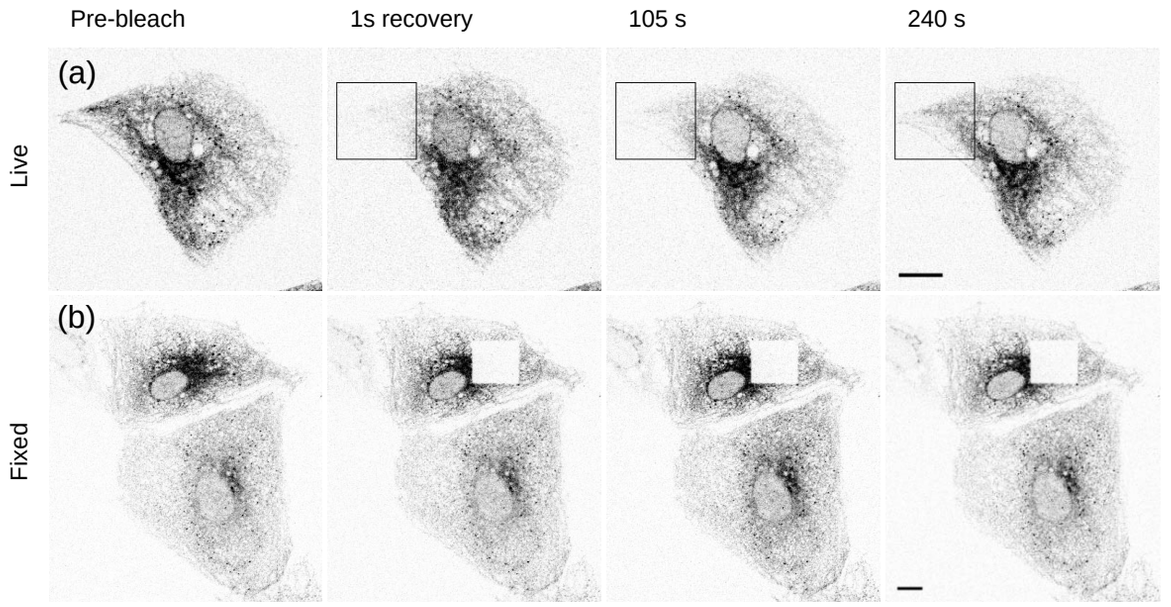

FIGURE 2

Qualitative fluorescence recovery after photobleaching (FRAP). A box was bleached in live or fixed PtK_2 cells expressing KDELR–GFP. (a) The indicated area was bleached at high laser power to approximately half of its original brightness and then recovery of unbleached KDELR–GFP into the bleach region was monitored with a low-power image every 2 s for 3 min. The bleached region recovered ~90% of its total pre-bleach fluorescence. Measuring the brightness of the entire cell before the bleach and after recovery showed that the total fluorescence intensity removed by the bleach was ~7%. Recovery shows that KDELR–GFP moves through the ER, either by diffusion or active transport. (b) Fixed cells bleached under the same conditions do not recover. The bleach region can be defined precisely with the confocal laser scanning microscope, and bleaching does not extend out of the selected area. Bar, 10 μm.

different organelles[19], for example, or changes in response to a stimulus or upon recruitment to a structure[8,20,21]. The mobile fraction can be estimated by quantitating the fluorescence in the bleached region after a long recovery time and comparing it with the pre-bleach fluorescence intensity, correcting for total fluorescence removed by the bleach[3]. Within the capabilities of the microscope, the shape and location of the bleached region should be appropriate for the topology of the GFP-labelled structures. The bleach parameters are determined empirically; attenuation ratios (the ratio of bleach to sampling intensity) from 300 to 15 000 have been used[3,9]. In FLIP experiments, repeated bleaching and imaging in a regular manner allows semiquantitative analysis (Fig. 1). A control cell can be included in the same field as the bleached cell to verify that the observed effects are from bleaching and not the low-intensity imaging illumination and to control for changes in focus over long imaging periods. Fig. 2b shows that bleaching is always confined to the selected region and does not affect nearby structures or cells, although intense scattered light can be observed by eye while bleaching.

To derive an effective diffusion coefficient from FRAP, the recovery of relative fluorescence intensity within the bleach region is plotted as a function of time (Fig. 3). D_{eff} can be derived by fitting a function to this curve. D_{eff} indicates the surface area randomly sampled by the fluorophore in a given time, thus its unit is area per time, usually $\mu m^2\ s^{-1}$. Photobleaching theory primarily describes solutions for finding D_{eff} from bleaching a small spot in the planar, uniform plasma membrane[2,17,22]. However, GFP fusion proteins can label complex, nonuniform three-dimensional (3-D) structures. Therefore, the actual surface area sampled by the GFP fusion protein over a 3-D structure might be much higher than its net movement projected in the plane of the image. A problem arises in relating the experimentally measured spreading of fluorescence through a complex structure, reflected by D_{eff}, to the real D, intrinsic to the diffusing species, which would be measured in an idealized planar membrane. To determine D_{eff} as accurately as possible, theoretical models need to be developed that take topology into account[23,24]. When the surface area sampled by the GFP fusion protein is higher than the bleach area visualized in the image, the intrinsic D is higher than the measured D_{eff}. D_{eff} is therefore a lower limit for the intrinsic D. Recovery might occur by more than one process – a membrane-bound GFP fusion protein could recover both by lateral diffusion in the plane of the membrane and by exchange with a cytosolic pool[19,25]. Flow- or tension-driven processes can also contribute to FRAP and can be distinguished from simple diffusion from the shape of the recovery curve[2,24].

GFP fusion proteins are considered to have a high mobility if they have both a high D_{eff} and a high mobile fraction. The mobile fraction indicates how much of the fluorophore is available for recovery. In practice, it is the ratio of the final to the initial fluorophore intensity in the bleach region, corrected for the amount of fluorophore removed by the bleach.

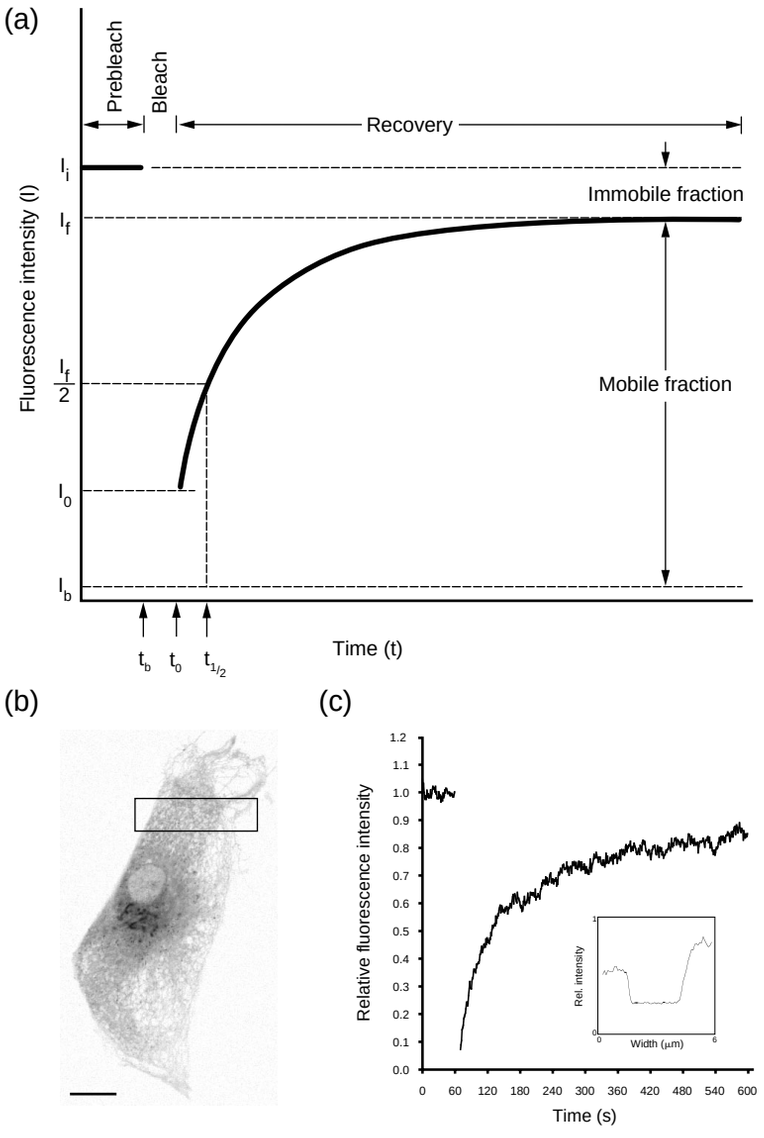

FIGURE 3

Quantitative fluorescence recovery after photobleaching (FRAP). (a) An idealized plot of fluorescence intensity (*I*) as a function of time shows the parameters of a quantitative FRAP experiment. The bleach region is monitored during a pre-bleach period to determine the initial intensity I_i. This region is bleached using high-intensity illumination from time t_b to t_0 (intensity is not plotted during the bleach), and recovery is monitored starting at t_0 until *I* reaches a final value I_f, when no further increase can be detected. Some methods calculate the effective diffusion coefficient, D_{eff}, directly from the time ($t_{1/2}$) to reach half final intensity ($I_f/2$; Ref. 2). To calculate D_{eff} as accurately as possible, *I* must be corrected for the background intensity, I_b, and the amount of total fluorescence removed by the bleach. The mobile fraction is the proportion of fluorescence that is regained, indicated by the difference between the initial and final fluorescence. (b) The region within the box was bleached across the endoplasmic reticulum (ER) of a live PtK_2 cell expressing KDELR–GFP and recovery of fluorescence recorded at 1 s intervals for 599 s. Bar, 10 μm. (c) The plot of I versus time for the bleaching experiment performed on the cell in (b). The inset shows the bleach profile averaged over the entire bleach region. D_{eff} was calculated from the data in (c) using the method described in Refs 15 and 24, which is an estimate of diffusion in one dimension (into the width of the box). Using this method, D_{eff} for KDELR–GFP in the ER is 0.413 $\mu m^2 s^{-1}$, with a mobile fraction of 86%. Images were analysed in NIH Image 1.62 and calculations of D_{eff} were performed in Mathematica 3.0. The estimated D_{eff} is a lower limit; to negotiate the complex network of the ER, KDELR–GFP has to move much farther in three dimensions to result in apparent net diffusion into the box in the plane of the image. Panel (a) adapted, with permission, from Ref. 15.

Applications of GFP photobleaching

Although D_{eff} is a lower limit, this limit is meaningful if there is a basis for comparison. Partikian and coworkers showed that the diffusion of GFP targeted to the mitochondrial matrix was not significantly different from the diffusion of the same GFP variant in water[6], indicating that the high protein density within the mitochondrial matrix did not significantly hinder mobility[9]. By comparison, a soluble GFP fusion protein targeted to a membrane-associated complex showed significantly slower recovery. Furthermore, the first application of GFP photobleaching showed that transmembrane GFP fusion proteins localized to the Golgi apparatus had surprisingly high lateral mobility[3]. The D_{eff} values for Golgi GFP proteins were some of the highest ever measured for transmembrane proteins. This indicated that immobilization is not a likely mechanism for their retention in the Golgi. Since D_{eff} is a lower limit of the intrinsic D, the GFP proteins in these studies could be even more mobile than measured.

Relative changes in the mobility of a GFP fusion protein are often as informative as absolute measures of diffusion. Such a change can correspond to assembly of a structure or complex. For example, a functional GFP fusion to E-cadherin (Ecad–GFP) was initially mobile within the membrane of nonpolarized cells[8]. When the cells polarized, Ecad–GFP was recruited into new sites of cell–cell contacts, with a corresponding reduction in mobile fraction and D_{eff}. When the contacts formed fully, Ecad–GFP became immobilized. A change in the mobility of a GFP fusion can also indicate disassembly of a structure. FRAP showed that a lamin-B receptor GFP fusion (LBR–GFP) exists in two pools in interphase cells: an immobile fraction assembled in the nuclear envelope (NE) and a highly mobile fraction in the endoplasmic reticulum (ER)[26]. During mitosis, the immobile NE fraction relocates to the ER, leaving only highly mobile LBR–GFP. FLIP experiments showed that, during interphase, the NE and ER fractions do not exchange rapidly, but, during mitosis, after the NE fraction has relocated to the ER, FLIP is able to deplete all GFP fluorescence.

Photobleaching of GFP–Arf1 shows how recovery can occur by multiple processes simultaneously. GFP–Arf1 cycles between a GTP-bound form associated with Golgi membranes, and a cytosolic GDP-bound form[25]. Photobleaching recovery correlates with GTP hydrolysis because a GFP–Arf1 mutant impaired in GTP hydrolysis (GFP–Arf1 Q71L) recovered almost fourfold more slowly than the wild-type GFP–Arf1. Since the mutant and nonmutant GFP–Arf1 proteins should diffuse laterally at the same rates, the authors concluded that, although diffusion did occur, exchange with the cytosolic pool limited recovery. In another study, Oancea and coworkers were able to differentiate between exchange and lateral diffusion of a Cys1–GFP that had translocated from the cytosol to the plasma or nuclear membrane in response to stimulation of diacylglycerol signalling[19]. They measured fluorescence recovery two ways: the expansion of the photobleached spot over time (a change in shape) and the increase in fluorescent

brightness within the photobleached region (a change in intensity). The expansion of the spot indicated lateral diffusion, and the increase in intensity reflected the sum of lateral diffusion and exchange between the cytosolic- and membrane-bound forms of the Cys1–GFP. By separating two contributions to photobleaching recovery (exchange and lateral diffusion), the authors were able to conclude that, when Cys1–GFP associated with membranes, its lateral mobility was also reduced.

Photobleaching GFP can demonstrate the continuity of intracellular organelles. FLIP experiments of Golgi-localized GFP fusion proteins provided the first evidence that a Golgi-localized protein could diffuse through the entire structure[3]. Similarly, Köhler and coworkers investigated the continuity of plastids in living plant cells with FLIP[27]. Plastids were thought to be autonomous organelles, but GFP fluorescence re-equilibrated between a bleached plastid and its neighbours, showing they are interconnected. Two further studies have examined the continuity of the ER. FRAP was used in both since the timescale required for the experiments was too short to bleach repeatedly and observe FLIP. Terasaki and coworkers targeted a GFP fusion (GFP–KDEL) to the ER lumen to visualize the ER in starfish eggs[20]. Before fertilization, GFP–KDEL diffused freely, with a high D_{eff} and high mobile fraction. Shortly after fertilization, ER continuity was disrupted such that bleached areas could not exchange with one another and thus no longer recovered quickly. A similar change in the continuity of the ER occurred in RBL cells when the level of cytosolic Ca^{2+} was increased, showing that the ER fragmented under these conditions[28]. Further FRAP experiments in RBL cells showed that the NE was continuous with the ER under quiescent conditions, but, upon Ca^{2+}-induced ER fragmentation, the NE remained intact.

GFP photobleaching reveals not only the mobility of the GFP fusion protein itself but also the mobility of entire intracellular structures labelled with GFP. For example, FRAP of GFP-labelled secretory granules in PC12 cells showed that only a fraction of the total granule population could move quickly enough to support regulated fusion and release[21].

Frequently, the brightness of excess GFP fusion protein obscures events of interest. Photobleaching provides a simple solution to this problem. Trafficking of GFP-labelled transport carriers in live neurons could only be observed after bleaching away a high GFP background[5]. Similarly, Presley and coworkers could only observe fusion of GFP-labelled transport carriers with the Golgi apparatus when they bleached away Golgi-accumulated fluorescence[4]. Before bleaching, the intensely bright Golgi obscured the fainter transport carriers.

A bright future for bleaching

Improvements in GFP technology in the past three years have developed concurrently with advances in fluorescence microscopy[18] and the availability of cost-effective computing resources to handle the large amounts of data produced by time-lapse digital microscopy. Such improvements make it convenient and meaningful to tag proteins with GFP. As a result, the potential applications of GFP photobleaching techniques are increasing dramatically. GFP photobleaching experiments could be used not only in tissue-culture cells to investigate intracellular events but also within tissues and even entire organisms; the technique is amenable to investigate the diffusion of GFP-tagged signalling molecules over a large field of cells. Additionally, the capability to double-label live cells with two different colour variants of fluorescent protein (see the article by Jan Ellenberg, Jennifer Lippincott-Schwartz and John Presley, in this issue[29]) allows the possibility to compare the diffusion of two different proteins simultaneously in the same living cell.

References

1 Tsien, R. and Waggoner, A. (1995) in *Handbook of Confocal Fluorescence Microscopy* (Pawley, J., ed.), pp. 267–277, Plenum Press
2 Axelrod, D. *et al.* (1976) *Biophys. J.* 16, 1055–1069
3 Cole, N. B. *et al.* (1996) *Science* 273, 797–801
4 Presley, J. F. *et al.* (1997) *Nature* 389, 81–85
5 Nakata, T., Terada, S. and Hirokawa, N. (1998) *J. Cell Biol.* 140, 659–674
6 Swaminathan, R., Hoang, C. P. and Verkman, A. S. (1997) *Biophys. J.* 72, 1900–1907
7 Patterson, G. H. *et al.* (1997) *Biophys. J.* 73, 2782–2790
8 Adams, C. L. *et al.* (1998) *J. Cell Biol.* 142, 1105–1119
9 Partikian, A. *et al.* (1998) *J. Cell Biol.* 140, 821–829
10 Yang, F., Moss, L. G. and Phillips, G. N., Jr (1996) *Nat. Biotechnol.* 14, 1246–1251
11 Ormo, M. *et al.* (1996) *Science* 273, 1392–1395
12 Yang, T. T., Cheng, L. and Kain, S. R. (1996) *Nucleic Acids Res.* 24, 4592–4593
13 Kneen, M. *et al.* (1998) *Biophys. J.* 74, 1591–1599
14 Llopis, J. *et al.* (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 6803–6808
15 Ellenberg, J. and Lippincott-Schwartz, J. (1998) in *Cells: a Laboratory Manual* (Spector, D., Goldman, R. and Leinwand, L., eds), pp. 79.71–79.23, Cold Spring Harbor Laboratory Press
16 Lippincott-Schwartz, J. *et al.* (1998) in *GFP Biofluorescence: Imaging Gene Expression and Protein Dynamics in Living Cells* (Vol. 58) (Sullivan, K. and Kay, S. A., eds), Methods in Cell Biology, Academic Press
17 Wolf, D. E. (1989) in *Fluorescence Microscopy of Living Cells in Culture* (Vol. 30, Part B) (Taylor, D. L. and Wang, Y., eds), pp. 271–332, Academic Press
18 Rizzuto, R., Carrington, W. and Tuft, R. A. (1998) *Trends Cell Biol.* 8, 288–292
19 Oancea, E. *et al.* (1998) *J. Cell Biol.* 140, 485–498
20 Terasaki, M. *et al.* (1996) *Dev. Biol.* 179, 320–328
21 Burke, N. V. *et al.* (1997) *Neuron* 19, 1095–1102
22 Soumpasis, D. M. (1983) *Biophys. J.* 41, 95–97
23 Olveczky, B. P. and Verkman, A. S. (1998) *Biophys. J.* 74, 2722–2730
24 Sciaky, N. *et al.* (1997) *J. Cell Biol.* 139, 1137–1155
25 Vasudevan, C. *et al.* (1998) *J. Cell Sci.* 111, 1277–1285
26 Ellenberg, J. *et al.* (1997) *J. Cell Biol.* 138, 1193–1206
27 Köhler, R. H. *et al.* (1997) *Science* 276, 2039–2042
28 Subramanian, K. and Meyer, T. (1997) *Cell* 89, 963–971
29 Ellenberg, J., Lippincott-Schwartz, J. and Presley, J. (1999) *Trends Cell Biol.* 9, 52–56

Acknowledgements

We gratefully thank Jan Ellenberg, Melissa Rolls, Frank Bradke and John Presley for critical comments on the manuscript, Stephan Grill for calculations in Mathematica, and James Nelson for communicating unpublished results.