INNOVATION

Photoactivatable fluorescent proteins

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Abstract | The fluorescence characteristics of photoactivatable proteins can be controlled by irradiating them with light of a specific wavelength, intensity and duration. This provides unique possibilities for the optical labelling and tracking of living cells, organelles and intracellular molecules in a spatio-temporal manner. Here, we discuss the properties of the available photoactivatable fluorescent proteins and their potential applications.

Genetically encoded fusions of cellular proteins with a green fluorescent protein from the jellyfish *Aequorea victoria* (avGFP; class hydrozoa) or with its multicolour homologues from class anthozoa have become indispensable tools in cell biology[1,2]. Such fusion proteins enable the non-invasive analysis of protein localization and dynamics in living cells owing to the unique ability of GFP-like fluorescent proteins and chromoproteins to form chromophores autocatalytically without the involvement of external enzymes and cofactors except for molecular oxygen[3]. Recently, a novel methodology has emerged with the development of so-called photoactivatable fluorescent proteins (PAFPs), which are capable of pronounced changes in their spectral properties in response to irradiation with light of a specific wavelength and intensity. Some PAFPs convert from a low (dark) to a bright fluorescent state (photoactivation), whereas others change fluorescence colour (photoswitching or photoconversion; FIG. 1a). Here, we use the general term photoactivation for all PAFPs, because their applications are always based on a marked increase in the fluorescent signal. PAFPs seem to be an excellent tool for the precise optical labelling and tracking of proteins, organelles and cells in living systems. They bring a new dimension to the kinetic microscopy of living cells, which was traditionally associated with fluorescence recovery after photobleaching (FRAP) approaches[1]. The PAFPs that have been developed so far can be divided into three main groups according to their mechanism of photoactivation — these groups are discussed below.

Interestingly, avGFP and some of its mutants are capable of light-induced transition from a green to a red fluorescent state in the absence of oxygen[4,5], although the mechanism of this GFP 'redding' remains unknown. However, this transition has limited applications, because it can only be used in facultative anaerobic organisms[6,7].

Among the PAFPs, there are obligate tetramers and monomeric proteins (TABLE 1). The main advantage of monomeric PAFPs is that they can be used to label and track individual intracellular molecules. Tetrameric PAFPs can be used to label whole cells and organelles but might disrupt the localization and function of their fusion partner. Two general approaches have recently been used to engineer monomeric PAFPs. First, photoactivatable properties have been introduced into monomeric chromoproteins or fluorescent proteins by mutating selected residues in the chromophore or its proximity[8,9,10]. Second, tetrameric PAFPs have been made monomeric by mutagenesis that disrupts the tetramer interfaces[11,12].

Properties of photoactivatable proteins

PA-GFP, PS-CFP and PAmRFP1. This group of PAFPs consists of a photoactivatable variant of avGFP known as PA-GFP[8], a photoswitchable cyan fluorescent protein, (PS-CFP)[9], which was developed from a GFP-like protein from the jellyfish *Aequorea coerulescens*, and PAmRFP1 proteins (REF. 10), which are red photoactivatable variants of a monomeric version[13] of DsRed from *Discosoma* sp.[14].

The excitation spectrum of avGFP consists of two peaks, one at 396 nm and the other at 476 nm. These peaks have a magnitude ratio of six to one, and they correspond to the neutral (protonated) and anionic (deprotonated) chromophore forms, respectively[15,16,17]. Ultraviolet (UV)–violet-light irradiation changes the ratio of the peaks slightly in favour of the anionic form[18]. It has been shown that these changes are brought about by Glu222 decarboxylation, which results in the rearrangement of the hydrogen-bonding network and chromophore deprotonation[19,20]. This basic mechanism apparently underlies the pronounced photoconversion of PA-GFP and PS-CFP (FIG. 1b).

PA-GFP is the result of a single residue substitution, Thr203His, in avGFP, which produces the mostly neutral chromophore form[8]. This form is characterized by 400-nm excitation and 515-nm emission maxima. Almost no fluorescence is observed when non-photoactivated PA-GFP is excited at wavelengths that correspond to the excitation spectrum of the anionic chromophore form (480–510 nm). Intense violet-light irradiation (at ~400 nm) causes irreversible photoconversion of the PA-GFP chromophore from the neutral to the anionic form, which absorbs at 504 nm and fluoresces at 517 nm (FIG. 1a). This photoconversion results in a 100-fold increase in the green fluorescence of the anionic form.

In contrast to PA-GFP, non-photoactivated PS-CFP fluoresces cyan, with emission peaked at 468 nm (REF. 9). The irreversible photoconversion of PS-CFP leads to a 300-fold increase

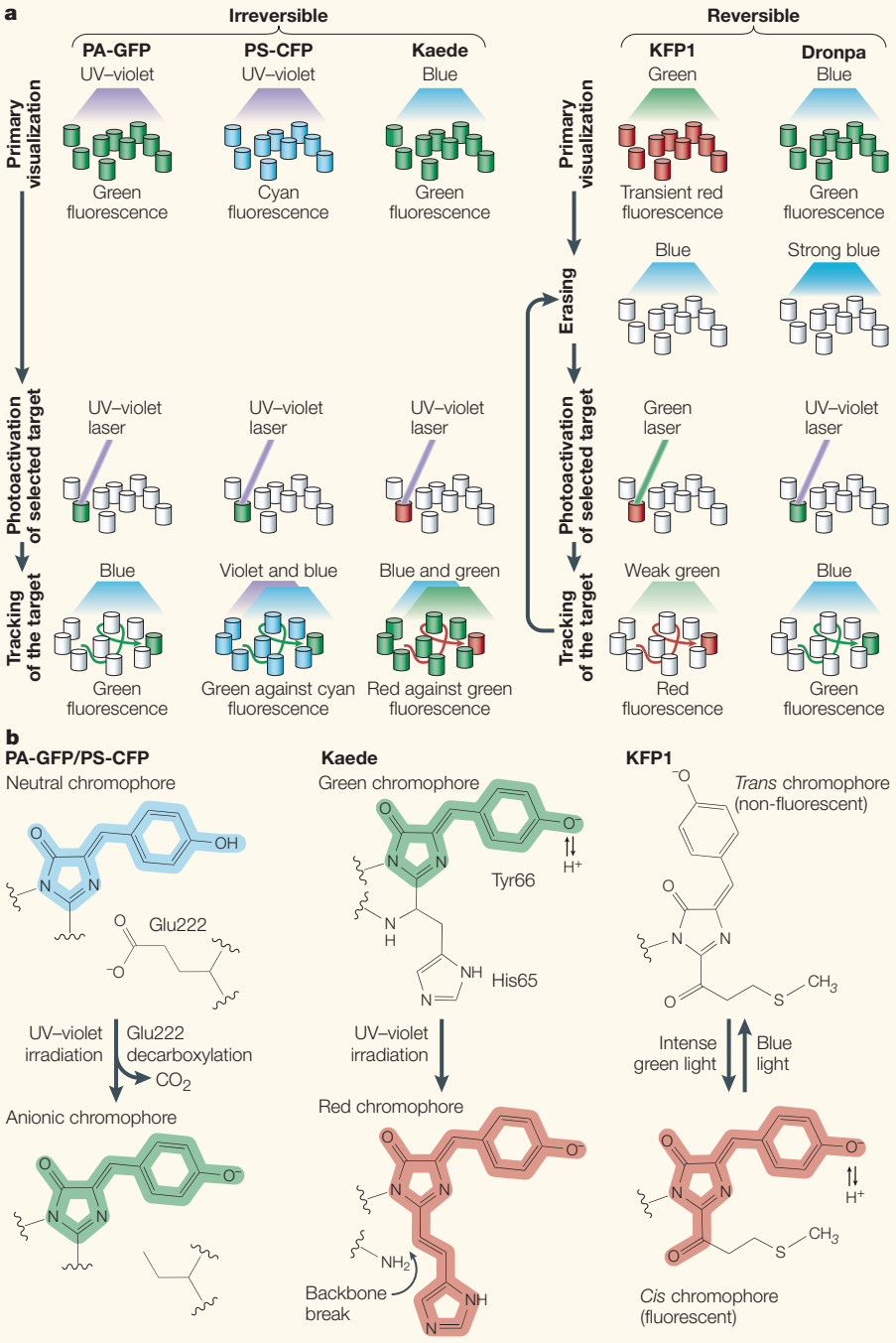


Figure 1 | **Spectral and photochemical properties of photoactivatable fluorescent proteins.** **a** | Procedures for selective photolabelling using irreversible and reversible photoactivatable fluorescent proteins (PAFPs). Cylinders represent an object, such as a cell, organelle or protein, that has been tagged with a PAFP, and the cylinders are coloured according to the emission colours before and after photoactivation. The excitation or activating light is shown above the cylinders as coloured trapezuims or lines respectively. Only reversible activation is shown for KFP1 for simplicity (KFP1 is capable of both reversible and irreversible photoactivation depending on the intensity and duration of the activating light). **b** | Chromophore photoconversion mechanisms for PA-GFP and PS-CFP (decarboxylation of Glu222 followed by chromophore conversion from a neutral to anionic state), Kaede (breakage of the polypeptide backbone and formation of an extra chromophore double bond), and KFP1 (probably *trans–cis* chromophore isomerization). The coloured shading of the chemical structures correspond to the spectral range of chromophore emission (PS-CFP in the left panel; the *trans* form of KFP1 is non-fluorescent in the right panel). The protonation–deprotonation equilibrium shown for Kaede and KFP1 is important for their photochemical behaviour. In both cases, the protonated chromophore can undergo photoconversion, whereas the deprotonated chromophore determines the fluorescent properties of the protein. KFP1, kindling fluorescent protein-1; PA-GFP, photoactivatable green fluorescent protein; PS-CFP, photoswitchable cyan fluorescent protein; UV, ultraviolet.

in green fluorescence at 511 nm and a 5-fold decrease in cyan fluorescence, which results in a 1,500-fold increase in the green-to-cyan fluorescence ratio (FIG. 1a). The drawback of PS-CFP is a relatively low brightness (a product of the excitation coefficient and the quantum yield) of both the initial cyan form and the photoactivated green form. In this respect, an enhanced PS-CFP2 variant, which is a commercially available version of PS-CFP, has been improved substantially (TABLE 1).

UV-induced photoactivation has also been observed for three PAmRFP1s variants[10] that were rationally designed on the basis of mRFP1 (REF. 13). PAmRFP1s have residue substitutions at positions 148, 165 and 203 compared to mRFP1. The brightest variant, PAmRFP1-1 (TABLE 1), initially has weak cyan fluorescence, but after its irreversible photoactivation at 380 nm it exhibits a 70-fold increase in red fluorescence with excitation and emission maxima at 578 nm and 605 nm, respectively.

Kaede and Kaede-like proteins. This group of PAFPs includes Kaede[21] (named after the Japanese maple leaf) from *Trachyphyllia geoffroyi*. It also includes Kaede-like proteins from anthozoa corals such as *Montastraea cavernosa* red fluorescent protein (mcavRFP)[22], *Ricordea florida* rfloRFP[22], *Lobophyllia hemprichii* EosFP[12] and its monomeric variant mEosFP[12] (named after the goddess of dawn in Greek mythology), and KikGR[23], a protein rationally engineered from the green fluorescent protein KikG (named after the Japanese name for this coral, kikume-ishi) from *Favia favus*.

In response to UV–violet-light irradiation which peaks at 380 nm, Kaede undergoes irreversible photoconversion from a green to a red fluorescent form (FIG. 1a). The resulting increase in the red-to-green fluorescence ratio after such photoactivation reaches 2,000-fold. Similar UV-activatable properties are observed for other Kaede-like proteins. The structural basis for such photoconversion is the unique positioning of His65. The chromophore in Kaede group members is formed by the tripeptide His65-Tyr66-Gly67 (the residue numbering relates to avGFP), which is similar to the avGFP Ser65-Tyr66-Gly67 chromophore. In response to UV–violet-light irradiation, the protein backbone in Kaede undergoes cleavage between the amide nitrogen and the Cα of His65 and a double bond forms between the Cα and Cβ of His65 (REF. 24). This extends the π-electron

Table 1 | **Comparison of the spectroscopic properties of selected photoactivatable fluorescent proteins (PFAPs)**

PAFP properties	PA-GFP	PS-CFP	PS-CFP2	PAmRFP1-1	Kaede	mEosFP	KikGR	KFP1*	Dronpa
Oligomeric state	Monomer‡	Monomer‡	Monomer‡	Monomer‡	Tetramer§	Monomer‡	Tetramer§	Tetramer§	Monomer‡
Activating light	UV–violet§	UV–violet§	UV–violet§	UV–violet§	UV–violet§	UV–violet§	UV–violet§	Green‡	UV–violet§
Quenching light	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Blue, max at ~450 nm	Blue, max at ~490 nm
Change of absorbance spectrum (nm)	400 to 504	402 to 490	400 to 490	Increase at 578	508 to 572	505 to 569	507 to 583	Increase at 590	Increase at 503
Change of emission spectrum (nm)	Increase at 517	468 to 511	470 to 511	Increase at 605	518 to 580	516 to 581	517 to 593	Increase at 600	Increase at 518
Reversibility of photoactivation	Irreversible	Irreversible	Irreversible	Irreversible	Irreversible	Irreversible	Irreversible	Reversible and irreversible‡	Reversible‡
Increase in fluorescence intensity (fold)	100	300‡	>400‡	70	800‡	ND	ND	70 or 35	ND
Fluorescence contrast (fold)	~200	1,500‡	>2,000‡	N/A	2,000‡	ND	>2,000‡	N/A	N/A
Before activation:QY	0.13	0.16	0.2	ND	0.88	0.64	0.7	<0.001	ND
Before activation: EC	20,700 at 400 nm	34,000 at 402 nm	43,000 at 400 nm	ND	98,800 at 508 nm	67,200 at 505 nm	28,200 at 507 nm	123,000 at 590 nm	ND
Before activation: pK_a	4.5‡	4.0‡	4.3‡	ND	5.6	ND	7.8	ND	ND
Before activation: brightness‖	0.08§	0.17§	0.26	ND	2.64‡	1.3‡	0.60‡	<0.004	ND
After activation: QY	0.79	0.19	0.23	0.08	0.33	0.62	0.65	0.07	0.85
After activation: EC	17,400 at 504 nm	27,000 at 490 nm	47,000 at 490 nm	10,000 at 578 nm	60,400 at 572 nm	37,000 at 569 nm	32,600 at 583 nm	59,000 at 590 nm	95,000 at 503 nm
After activation: pK_a	ND	6.0	6.1	4.4‡	5.6‡	ND	5.5‡	ND	5.0‡
After activation: brightness‖	0.42	0.16§	0.33	0.03§	0.60‡	0.70‡	0.64‡	0.13§	2.45‡
Source organism (class)	*Aequorea victoria* (hydrozoa)	*Aequorea coerulescens* (hydrozoa)	*Aequorea coerulescens* (hydrozoa)	*Discosoma* spp. (anthozoa)	*Trachyphyllia geoffroyi* (anthozoa)	*Lobophyllia hemprichii* (anthozoa)	*Favia favus* (anthozoa)	*Anemonia sulcata* (anthozoa)	*Pectiniidae* spp. (anthozoa)
Reference	8	9	–	10	22	12	23	25	11
Commercially available	No	No	Yes, Evrogen	No	Yes, MBL Intl	No	Yes, MBL Intl	Yes, Evrogen	Yes, MBL Intl

*The QY (quantum yield) and EC (extinction coefficient ($M^{-1}cm^{-1}$)) shown are for irreversibly photoactivated KFP1. ‡The main advantages of the PAFPs. §The main disadvantages of the PAFPs. ‖Intrinsic brightness is a product of QY and EC; the brightness values are normalized to the intrinsic brightness of EGFP[3]. EGFP, enhanced variant of *Aequorea victoria* green fluorescent protein; KFP1, kindling fluorescent protein-1; KikGR, a protein engineered from KikG from *Favia favus*; max, maximum; MBL Intl, Medical and Biological Laboratories Corporation International; mEosFP, monomeric Eos fluorescent protein; N/A, not applicable; ND, not determined; PAFP, photoactivatable fluorescent protein; PA-GFP, photoactivatable green fluorescent protein; PAmRFP1-1, photoactivatable monomeric red fluorescent protein-1-1; PS-CFP, photoswitchable cyan fluorescent protein; UV, ultraviolet.

conjugation of Tyr66 to the imidazole ring of His65 and results in a red shift of Kaede fluorescence (FIG. 1b).

Kaede group members represent excellent photoactivatable markers for labelling organelles and cells. Although most of these PAFPs are tetrameric and are therefore unsuitable for fusion-protein studies, a monomeric variant, known as mEosFP, has been recently developed[13] (TABLE 1). However, chromophore formation in mEosFP occurs only at temperatures below 30°C, which limits its applications in mammalian cells.

KFP and Dronpa. This group of anthozoa-derived proteins includes reversible PAFPs — kindling fluorescent protein-1 (KFP1)[25,26], which was developed from the *Anemonia sulcata* chromoprotein asulCP (also known as asFP595 and asCP), and Dronpa (named after the Japanese word meaning

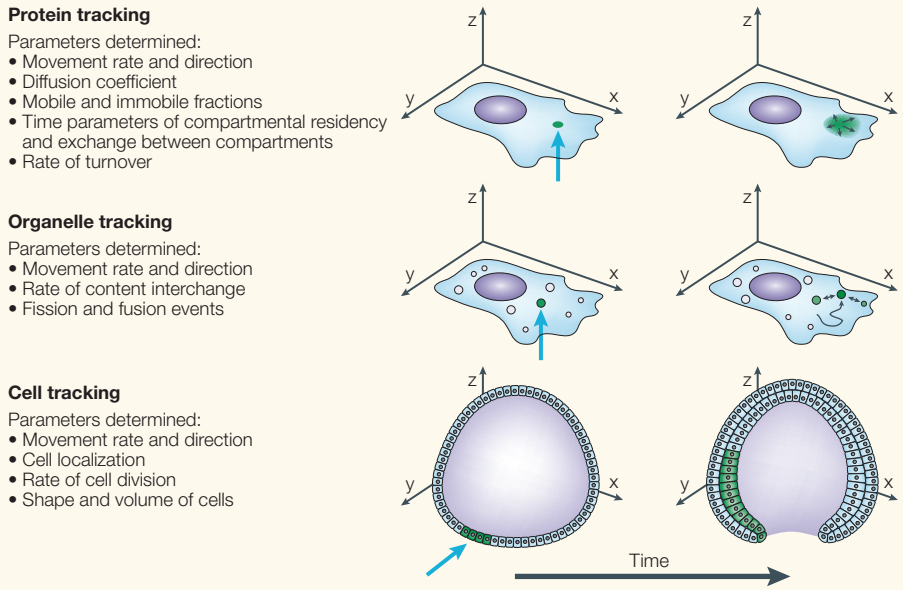


Figure 2 | **Three levels of spatio-temporal labelling with photoactivatable fluorescent proteins.** A focused beam of light (blue arrows) is used to activate photoactivatable fluorescent proteins (green zones) in a region of interest in a cell, tissue or organism. Migration of the labelled object (protein, organelle or cell) can then be monitored over time. A number of qualitative and quantitative parameters can be determined in each case.

disappearance of a ninja) from *Pectiniidae* sp. coral[11].

Wild-type asulCP converts to a red fluorescent form (excitation and emission spectra peaks at 572 and 595 nm, respectively) in response to irradiation with intense green light. This fluorescent state is unstable in asulCP, so in the dark it spontaneously relaxes back to a non-fluorescent form within a minute[27]. This type of photochemical behaviour was termed 'kindling'. On the other hand, blue light which peaks at 450 nm instantly quenches asulCP back to its non-fluorescent form. Both photoactivation and quenching of asulCP are reversible processes. The enhanced asulCP mutant, KFP1, is capable of both reversible and irreversible photoactivation depending on the intensity and duration of the activating light[25,26] (see FIG. 1a, which shows its reversible photoactivation).

A PAFP that is capable of reversible photoconversion from a green to non-fluorescent form, known as Dronpa[11], has also been developed (FIG. 1a). Initially, Dronpa fluoresces green with excitation and emission spectra that peak at 503 nm and 518 nm, respectively (TABLE 1). Prolonged or intense irradiation with blue light (470–510 nm) leads to protein quenching to a non-fluorescent neutral chromophore form that has an absorption maximum at about 390 nm. Dronpa can then be reversibly activated back to a fluorescent form by irradiation at 400 nm. Remarkably, these activation–quenching events can be repeated many times in a single cell for KFP1 and Dronpa.

The photochemical behaviour of KFP1 and Dronpa might be similar. Both proteins are capable of repeated photoconversion between two states using light of two distinct wavelengths. For Dronpa, these wavelengths correspond to neutral and anionic chromophore forms, and the same chromophore forms have been proposed for KFP1 (REF. 26). A *trans–cis* chromophore isomerization was proposed to explain the reversible photoactivation of KFP1 (REFS 26,28,29) (FIG. 1b), and the same mechanism might underlie Dronpa photoactivation. However, a detailed understanding of this phenomenon requires further study.

Applications of photoactivation

Pulse-chase photolabelling and tracking. The three main levels of PAFP application are protein, organelle and cell labelling (FIG. 2). In contrast to the observation of fluorescently tagged objects by constant imaging, PAFPs allow the object to be tracked without the need for continual visualization. This feature greatly extends the spatio-temporal limits of studies of biological dynamics, and reduces the photobleaching and phototoxicity problems of imaging procedures. Many examples of the potential applications of PAFPs that are listed in TABLE 2 have not yet been realized because of the novelty of photoactivation approaches, but we anticipate that they will be explored in the near future.

PAFPs provide a unique opportunity for the non-invasive labelling and tracking of specific cell types in living organisms and tissues that show considerable cellular movement. Obvious examples include the study of embryogenesis, metastasis and tumour formation, the migration of small parasites within a host, and the taxis reactions of free unicellular organisms. Various cellular organelles can also be loaded with PAFPs through the use of polypeptide targeting signals or through their fusion to proteins that have a specific subcellular localization. PAFPs provide the opportunity to study the transport, fusion and fission events of individual organelles.

Perhaps the most important application of PAFPs is protein labelling. When fused to a protein of interest, PAFPs can provide detailed information about protein localization, turnover, and the direction and rate of trafficking in a living cell[1]. For example, a PA-GFP–α-tubulin fusion construct allowed the movement of peripheral microtubules and their inclusion in a mitotic spindle to be observed[30]. DNA and RNA molecules can also be labelled with PAFPs and tracked. This labelling involves an interaction between a specific DNA/RNA-binding domain that is fused to a PAFP and the corresponding target sequence that can be introduced into the nucleic acid of interest[31]. It would be useful to apply PAFPs to, for example, the visualization of protein and organelle exchange between desmosome-connected plant cells, as well as to mammalian cells that are connected by the recently discovered membrane nanotubes[32,33]. By using PAFPs with distinct colours, it should be possible to study the dynamics of several proteins simultaneously.

In contrast to irreversibly photoactivatable probes, reversible PAFPs allow repeated activation events and the photolabelling of several subcellular regions one after another. Ando and co-workers monitored the nuclear import and export of extracellular signal-regulated kinase-1 (ERK1), as well as changes in the rate of ERK1 transport in response to cell stimulation, using the repeated photoactivation and erasing of a Dronpa–ERK1 signal in the nucleus and cytoplasm of a single cell[11]. This feature of reversible PAFPs should result in a novel approach to map protein-trafficking pathways and to reveal the 'topography' of subcellular transportation and signalling.

Table 2 | ***In vivo* applications for photoactivatable fluorescent proteins**

Tracked object	Biological problem or application	References
Cells in a tissue or organism	Tracking cells during development	25
	Tracking lymphocytes in inflammation studies	-
	Tracking cells in cancer and metastasis	-
	Tracking unicellular organisms, free living and in a host	-
	Visualizing individual cells of intricate shapes (for example, neurons) in a complex network	22
Cellular organelles or vesicles	Visualizing exchange with the extracellular space, such as endocytosis, exocytosis and phagocytosis	9,50
	Studying intracellular organelle continuity, sorting, fusion and fission events	7,25,50–52
	Following the exchange of organelle content	8–10
	Visualizing membrane diffusion and fluidity	-
Molecules in a living cell	Studying intracellular diffusion	6,8,11,53
	Studying intracellular directional transport	9,11,53
	Monitoring the assembly/disassembly of, for example, the cytoskeleton or nucleoli	30,54
	Studying post-translational-modification and degradation pathways	–
	Investigating intercellular exchange	–
	Tracking viruses or protein particles in a host	55
	Visualizing RNA and DNA dynamics	31
	Carrying out photoactivatable and photochromic fluorescence-resonance-energy-transfer experiments	–

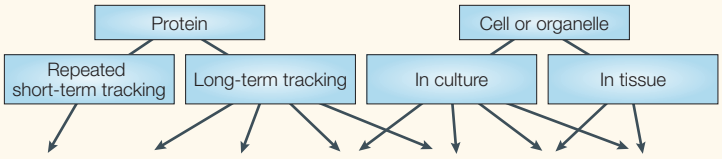
The rapidly growing number of PAFPs that are available raises a question: which PAFP should you select to achieve the best results? Each group of PAFPs has particular features that make it preferable for certain types of application. Criteria for PAFP selection that depend on the experiment being undertaken and the required spectral properties are shown in FIG. 3.

Detection of protein interactions. PAFPs open new avenues for the study of protein–protein interactions using fluorescence resonance energy transfer (FRET). FRET is a non-radiative transfer of energy from one excited fluorophore, the donor, to another fluorophore, the acceptor, which is in close proximity to the donor and has an excitation spectrum that overlaps with the donor emission[34]. FRET results in the fluorescence of the acceptor when the donor is excited by external light. Using an irreversible PAFP as a donor, acceptor or both in photoactivatable FRET (PA-FRET) microscopy would make it possible to study the spatio-temporal localization and interaction of fusion proteins simultaneously. If FRET is detected between a PAFP donor and a fluorescent protein acceptor in one cellular compartment after photoactivation in a different cellular region, this might indicate that the target protein fused to the PAFP moves between these two locations.

Recent advances in FRET imaging[35,36] mean that there are FRET approaches for which PAFPs could be particularly useful. Specifically, photochromic FRET (PC-FRET) that uses organic dyes with reversibly photoswitchable absorbance spectra as acceptors has been described[37,38]. Reversible PAFPs such as KFP1 and Dronpa might therefore find their use in PC-FRET microscopy. Their absorbance is substantially changed following photoactivation, which provides an excellent inner control for FRET detection. If the intensity or lifetime of the donor fluorescence is affected by PAFP acceptor photoswitching, this is indicative of the proximity of its partner. Moreover, a PC-FRET pair can be used as a benchmark for determining intermolecular distances. With knowledge of the overlap integrals for the donor excitation and acceptor absorbance for both states of a PAFP acceptor, two independently measured FRET efficiencies will allow the exclusion of other parameters from the FRET equation[34] and the calculation of an average distance between the interacting proteins. A recently developed CFP, known as Cerulean[39], and Dronpa seem to be a good PC-FRET pair. Indeed, the Cerulaen emission (maximum at 475 nm) and Dronpa excitation (maximum at 503 nm) curves overlap significantly, which ensures a high FRET efficiency. At the same time, the spectral overlap (and therefore the FRET efficiency) should decrease markedly after Dronpa quenching, because its excitation peak at 503 nm falls almost to zero. Importantly, Dronpa quenching using 488-nm light should not result in undesirable Cerulean bleaching (its absorption maximum is at 433 nm). However, a potential problem could be a fast reactivation of the quenched Dronpa by the light that is used to excite Cerulean.

An approach to photoactivate PAFPs through the prolonged, accumulated action of donor emission could be developed with a FRET pair that consists of a luciferase donor and an irreversible PAFP acceptor. If the luciferase luminescence activates a nearby PAFP, the degree of PAFP photoactivation will be proportional to the FRET efficiency and the time period during which the FRET pair interact. Owing to the accumulation of photoactivation, this approach would enable the detection of weak protein–protein interactions or of the history of an interaction in a cell. It would also allow the visualization of the spatial pattern of a protein–protein interaction in tissues and organisms, as the cells in which it occurred would be fluorescent. At present, the choice of luciferase and PAFP is restricted by the spectral properties of the available monomeric PAFPs, which are mostly sensitive to UV–violet light. Therefore, blue-shifted variants of luciferase that emit at 400 nm (REF. 40) are suitable for this assay.

Cellular imaging and sorting. An important area of application for reversible PAFPs is in high-resolution fluorescence imaging. Improving the spatial resolution of fluorescence microscopy is important for numerous applications. However, the wave-like nature of light imposes a diffraction limit of ~200 nm on fluorescence microscopy. A new concept known as stimulated emission depletion (STED), which breaks the diffraction limit of light microscopy, has recently been proposed[41]. The concept relies on the



	Dronpa	PAmRFP1	mEosFP	PA-GFP	PS-CFP2	Kaede	KFP1
	Monomer	Monomer	Monomer	Monomer	Monomer	Tetramer	Tetramer
	Reversible	Irreversible	Irreversible	Irreversible	Irreversible	Irreversible	(Ir)reversible
Fluorescence changes during photoactivation							
High brightness	✓			✓		✓	
High contrast			✓		✓	✓	
Dual labelling with red and green fluorescent proteins	✓	✓		✓	✓		✓
Low phototoxicity of the activation light							✓

Figure 3 | **Criteria for selecting photoactivatable fluorescent proteins for a particular tracking experiment.** The choice depends on the experiment being undertaken (upper blue rectangles) and the desired spectral features (lower tabulated information). In the row depicting fluorescence changes during photoactivation, the colouring from left to right indicates the fluorescence changes for each photoactivatable fluorescent protein (PAFP) during the course of their photoactivation. Monomeric photoactivable proteins can be used for protein tagging, whereas organelles and cells can be also labelled with tetrameric PAFPs. Prolonged protein tracking should be carried out using irreversible PAFPs. Reversible photoactivation might be preferable for short-term dynamics. Most PAFPs require phototoxic UV–violet-light photoactivation, and a higher power of light is required for the photoactivation of PA-GFP, PS-CFP2 and PAmRFP1s. A pulse of UV–violet light might cause substantial abnormalities during long-term studies of whole organisms. For example, a negative influence of the 408-nm light that was used to activate PA-GFP-tagged histone-2A on nuclei division in *Drosophila melanogaster* embryos has been observed[49]. This phototoxicity problem could be solved by multiphoton photoactivation with infrared lasers. Alternatively, KFP1, which is capable of both reversible and irreversible photoactivation depending on the intensity and duration of the activating light, provides the opportunity to use green light with a low phototoxicity. KFP1, kindling fluorescent protein-1; mEosFP, monomeric Eos fluorescent protein; PA-GFP, photoactivatable green fluorescent protein; PAmRFP1, photoactivatable monomeric red fluorescent protein-1; PS-CFP2, photoswitchable cyan fluorescent protein-2.

visualization of an extremely small spot of fluorescence probes that are initially excited with a laser and are immediately quenched in a doughnut-like pattern using a second, powerful laser. The fluorescent area that remains in the centre has a sub-diffraction size of tens of nanometres. Using this approach, reversible PAFPs that can undergo numerous light-induced transitions between dark and fluorescent states could possibly be used for live-cell imaging with a high spatial resolution at a low light intensity[42].

PAFPs could also contribute to fluorescence-activated cell-sorting techniques, functioning as the extra colours that can be turned on to further differentiate between cell types. For example, a combination of a GFP, an RFP and photoactivatable Kaede would allow the sorting of three cell types in two steps. First, the GFP- and Kaede-expressing cells could be separated from the RFP-expressing ones. UV–violet-light irradiation of the remaining green cell sample would lead to Kaede photoactivation from its green-to-red fluorescent state. These red cells could then be separated from the GFP-expressing cells in a second sorting step.

Future highlights

In the future, fluorescence microscopy will be able to spectrally resolve many colours in a cell using spectral imaging and linear unmixing approaches[43]. Therefore, advanced genetic engineering and molecular evolution strategies will be applied to develop multicolour PAFPs using monomeric fluorescent proteins and chromoproteins. For example, by using improved variants of mRFP1 (REFS 44,45) or GFP-like proteins using novel natural sources[46] as the templates. The desired PAFPs will undergo photoswitching at new wavelengths of non-toxic visible light, will result in photoactivated fluorescence in all areas of the visible spectrum and, moreover, will extend optical highlighting into the far-red region, in which tissue turbidity and cellular autofluorescence are low[47].

Molecular biosensors that consist of avGFP variants fused to 'sensitive' domains, such as specific binding peptides or scaffolds, recently made significant progress[48]. However, avGFP-based fusions have a substantial drawback, for example, a low range of fluorescence contrast. In this respect, PAFPs, with their ability to markedly change their fluorescent intensity, represent promising templates for the next generation of biosensors. Our results[10] (D.M.C., K.A.L. and V.V.V., unpublished data) show that in addition to light irradiation, the partial denaturation of some PAFPs by extremes of pH or urea can result in the chromophore converting between its dark and fluorescent state. So, combinations of PAFPs fused to sensitive domains might result in highly contrasting biosensors that can exhibit fluorescence changes of two orders of magnitude and can therefore allow the spatio-temporal visualization of extremely low levels of intracellular signalling.

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1. Lippincott-Schwartz, J. & Patterson, G. H. Development and use of fluorescent protein markers in living cells. *Science* **300**, 87–91 (2003).
2. Verkhusha, V. V. & Lukyanov, K. A. The molecular properties and applications of *Anthozoa* fluorescent proteins and chromoproteins. *Nature Biotechnol.* **22**, 289–296 (2004).
3. Tsien, R. Y. The green fluorescent protein. *Annu. Rev. Biochem.* **67**, 509–544 (1998).
4. Elowitz, M. B., Surette, M. G., Wolf, P. E., Stock, J. & Leibler, S. Photoactivation turns green fluorescent protein red. *Curr. Biol.* **7**, 809–812 (1997).
5. Sawin, K. E. & Nurse, P. Photoactivation of green fluorescent protein. *Curr. Biol.* **7**, 606–607 (1997).
6. Elowitz, M. B., Surette, M. G., Wolf, P. E., Stock, J. B. & Leibler, S. Protein mobility in the cytoplasm of *Escherichia coli*. *J. Bacteriol.* **181**, 197–203 (1999).
7. Jakobs, S., Schauss, A. C. & Hell, S. W. Photoconversion of matrix targeted GFP enables analysis of continuity and intermixing of the mitochondrial lumen. *FEBS Lett.* **554**, 194–200 (2003).
8. Patterson, G. H. & Lippincott-Schwartz, J. A photoactivatable GFP for selective photolabeling of proteins and cells. *Science* **13**, 1873–1877 (2002).
9. Chudakov, D. M. *et al.* Photoswitchable cyan fluorescent protein for protein tracking. *Nature Biotechnol.* **22**, 1435–1439 (2004).
10. Verkhusha, V. V. & Sorkin, A. Conversion of the monomeric red fluorescent protein into a photoactivatable probe. *Chem. Biol.* **12**, 279–285 (2005).

11. Ando, R., Mizuno, H. & Miyawaki, A. Regulated fast nucleocytoplasmic shuttling observed by reversible protein highlighting. *Science* **306**, 1370–1373 (2004).
12. Wiedenmann, J. *et al.* EosFP, a fluorescent marker protein with UV-inducible green-to-red fluorescence conversion. *Proc. Natl Acad. Sci. USA* **101**, 15905–15910 (2004).
13. Campbell, R. E. *et al.* A monomeric red fluorescent protein. *Proc. Natl Acad. Sci. USA* **99**, 7877–7882 (2002).
14. Matz, M, V. *et al.* Fluorescent proteins from nonbioluminescent *Anthozoa* species. *Nature Biotechnol.* **17**, 969–973 (1999).
15. Chattoraj, M., King, B. A., Bublitz, G. U. & Boxer, S. G. Ultra-fast excited state dynamics in green fluorescent protein: multiple states and proton transfer. *Proc. Natl Acad. Sci. USA* **93**, 8362–8367 (1996).
16. Niwa, H. *et al.* Chemical nature of the light emitter of the *Aequorea* green fluorescent protein. *Proc. Natl Acad. Sci. USA* **93**, 13617–13622 (1996).
17. Brejc, K. *et al.* Structural basis for dual excitation and photoisomerization of the *Aequorea victoria* green fluorescent protein. *Proc. Natl Acad. Sci. USA* **94**, 2306–2311 (1997).
18. Yokoe, H. & Meyer, T. Spatial dynamics of GFP-tagged proteins investigated by local fluorescence enhancement. *Nature Biotechnol.* **14**, 1252–1256 (1996).
19. Van Thor, J. J., Gensch, T., Hellingwerf, K. J. & Johnson, L. N. Phototransformation of green fluorescent protein with UV and visible light leads to decarboxylation of glutamate 222. *Nature Struct. Biol.* **9**, 37–41 (2002).
20. Bell, A. F., Stoner-Ma, D., Wachter, R. M. & Tonge, P. J. Light-driven decarboxylation of wild-type green fluorescent protein. *J. Am. Chem. Soc.* **125**, 6919–6926 (2003).
21. Ando, R., Hama, H., Yamamoto-Hino, M., Mizuno, H. & Miyawaki, A. An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein. *Proc. Natl Acad. Sci. USA* **99**, 12651–12656 (2002).
22. Labas, Y. A. *et al.* Diversity and evolution of the green fluorescent protein family. *Proc. Natl Acad. Sci. USA* **99**, 4256–4261 (2002).
23. Tsutsui, H., Karasawa, S., Shimizu, H., Nukina, N. & Miyawaki, A. Semi-rational engineering of a coral fluorescent protein into an efficient highlighter. *EMBO Rep.* **6**, 233–238 (2005).
24. Mizuno, H. *et al.* Photo-induced peptide cleavage in the green-to-red conversion of a fluorescent protein. *Mol. Cell* **12**, 1051–1058 (2003).
25. Chudakov, D. M. *et al.* Kindling fluorescent proteins for precise *in vivo* photolabeling. *Nature Biotechnol.* **21**, 191–194 (2003).
26. Chudakov, D. M., Feofanov, A. V., Mudrik, N. N., Lukyanov, S. & Lukyanov, K. A. Chromophore environment provides clue to 'kindling fluorescent protein' riddle. *J. Biol. Chem.* **278**, 7215–7219 (2003).
27. Lukyanov, K. A. *et al.* Natural animal coloration can be determined by a nonfluorescent green fluorescent protein homolog. *J. Biol. Chem.* **275**, 25879–25882 (2000).
28. Quillin, M. L. *et al.* Kindling fluorescent protein from *Anemonia sulcata*: dark-state structure at 1.38-A resolution. *Biochemistry* **44**, 5774–5787 (2005).
29. Wilmann, P. G., Petersen, J., Devenish, R. J., Prescott, M. & Rossjohn, J. Variations on the GFP chromophore: a polypeptide fragmentation within the chromophore revealed in the 2.1-A crystal structure of a nonfluorescent chromoprotein from *Anemonia sulcata*. *J. Biol. Chem.* **280**, 2401–2404 (2005).
30. Tulu, U. S., Rusan, N. M. & Wadsworth, P. Peripheral, non-centrosome-associated microtubules contribute to spindle formation in centrosome-containing cells. *Curr. Biol.* **13**, 1894–1899 (2003).
31. Shav-Tal, Y. *et al.* Dynamics of single mRNPs in nuclei of living cells. *Science* **304**, 1797–1800 (2004).
32. Rustom, A., Saffrich, R., Markovic, I., Walther, P. & Gerdes, H. H. Nanotubular highways for intercellular organelle transport. *Science* **303**, 1007–1010 (2004).
33. Onfelt, B., Nedvetzki, S., Yanagi, K. & Davis, D. M. Cutting edge: membrane nanotubes connect immune cells. *J. Immunol.* **173**, 1511–1513 (2004).
34. Lakowicz, J. R. in *Principles of Fluorescence Spectroscopy.* 291–318 (Kluwer Academic/Plenum New York, Boston, Dorbrech, London, Moscow,1999).
35. Jares-Erijman, E. A. & Jovin, T. M. FRET imaging. *Nature Biotechnol.* **21**, 1387–1395 (2003).
36. Galperin, E., Verkhusha, V. V. & Sorkin, A. Three-chromophore FRET microscopy to analyze multiprotein interactions in living cells. *Nature Methods* **1**, 209–217 (2004).
37. Giordano, L., Jovin, T. M., Irie, M. & Jares-Erijman, E. A. Diheteroarylethenes as thermally stable photoswitchable acceptors in photochromic fluorescence resonance energy transfer (pcFRET). *J. Am. Chem. Soc.* **124**, 7481–7489 (2002).
38. Song, L., Jares-Erijmana, E. A. & Jovin, T. M. A photochromic acceptor as a reversible light-driven switch in fluorescence resonance energy transfer (FRET). *J. Photochem. Photobiol.* **150**, 177–185 (2002).
39. X. Rizzo, M. A., Springer, G. H., Granada, B. & Piston, D. W. An improved cyan fluorescent protein variant useful for FRET. *Nature Biotechnol.* **22**, 445–449 (2004).
40. Deng, L. *et al.* Structural basis for the emission of violet bioluminescence from a W92F obelin mutant. *FEBS Lett.* **506**, 281–285 (2001).
41. Hell, S. W. Toward fluorescence nanoscopy. *Nature Biotechnol.* **21**, 1347–1355 (2003).
42. Hell, S. W., Dyba, M. & Jakobs, S. Concepts for nanoscale resolution in fluorescence microscopy. *Curr. Opin. Neurobiol.* **14**, 599–609 (2004).
43. Zimmermann, T., Rietdorf, J. & Pepperkok, R. Spectral imaging and its applications in live cell microscopy. *FEBS Lett.* **546**, 87–92 (2003).
44. Shaner, N. C. *et al.* Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nature Biotechnol.* **22**, 1567–1572 (2004).
45. Wang, L., Jackson, W. C., Steinbach, P. A. & Tsien, R. Y. Evolution of new nonantibody proteins via iterative somatic hypermutation. *Proc. Natl Acad. Sci. USA* **101**, 16745–16749 (2004).
46. Shagin, D. A. *et al.* GFP-like proteins as ubiquitous metazoan superfamily: evolution of functional features and structural complexity. *Mol. Biol. Evol.* **21**, 841–850 (2004).
47. Billinton, N. & Knight, A. W. Seeing the wood through the trees: a review of techniques for distinguishing green fluorescent protein from endogenous autofluorescence. *Anal. Biochem.* **291**, 175–197 (2001).
48. Griesbeck, O. Fluorescent proteins as sensors for cellular functions. *Curr. Opin. Neurobiol.* **14**, 636–641 (2004).
49. Post, J. N., Lidke, K. A., Rieger, B. & Arndt-Jovin, D. J. One- and two-photon photoactivation of a paGFP-fusion protein in live *Drosophila* embryos. *FEBS Lett.* **579**, 325–330 (2005).
50. Lee, W. L., Kim, M. K., Schreiber, A. D. & Grinstein, S. Role of ubiquitin and proteasomes in phagosome maturation. *Mol. Biol. Cell* **16**, 2077–2090 (2005).
51. Karbowski, M. *et al.* Quantitation of mitochondrial dynamics by photolabeling of individual organelles shows that mitochondrial fusion is blocked during the Bax activation phase of apoptosis. *J. Cell Biol.* **164**, 493–499 (2004).
52. Arimura, S., Yamamoto, J., Aida, G. P., Nakazono, M. & Tsutsumi, N. Frequent fusion and fission of plant mitochondria with unequal nucleoid distribution. *Proc. Natl Acad. Sci. USA* **101**, 7805–7808 (2004).
53. Deryusheva, S. & Gall, J. G. Dynamics of coilin in Cajal bodies of the *Xenopus* germinal vesicle. *Proc. Natl Acad. Sci. USA* **101**, 4810–4814 (2004).
54. Rusan, N. M. & Wadsworth, P. Centrosome fragments and microtubules are transported asymmetrically away from division plane in anaphase. *J. Cell Biol.* **168**, 21–28 (2005).
55. Stanek, D. & Neugebauer, K. M. Detection of snRNP assembly intermediates in Cajal bodies by fluorescence resonance energy transfer. *J. Cell Biol.* **166**, 1015–1025 (2004).

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OPINION

What is the role of protein aggregation in neurodegeneration?

Christopher A. Ross and Michelle A. Poirier

Abstract | Neurodegenerative diseases typically involve deposits of inclusion bodies that contain abnormal aggregated proteins. Therefore, it has been suggested that protein aggregation is pathogenic. However, several lines of evidence indicate that inclusion bodies are not the main cause of toxicity, and probably represent a cellular protective response. Aggregation is a complex multi-step process of protein conformational change and accretion. The early species in this process might be most toxic, perhaps through the exposure of buried moieties such as main chain NH and CO groups that could serve as hydrogen bond donors or acceptors in abnormal interactions with other cellular proteins. This model implies that the pathogenesis of diverse neurodegenerative diseases arises by common mechanisms, and might yield common therapeutic targets.

Neurodegenerative diseases, such as Parkinson's disease (PD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS) and the polyglutamine diseases that include Huntington's disease (HD), arise from abnormal protein interactions in the central nervous system. In all of these diseases, there are characteristic deposits of protein aggregates[1–11] in the brain, which can be cytoplasmic, nuclear or extracellular (FIG. 1). Protein