Photoswitchable fluorescent proteins: ten years of colorful chemistry and exciting applications

Xin X Zhou[1] and Michael Z Lin[1,2]

Reversibly photoswitchable fluorescent proteins (RSFPs) are fluorescent proteins whose fluorescence, upon excitation at a certain wavelength, can be switched on or off by light in a reversible manner. In the last 10 years, many new RSFPs have been developed and novel applications in cell imaging discovered that rely on their photoswitching properties. This review will describe research on the mechanisms of reversible photoswitching and recent applications using RSFPs. While cis–trans isomerization of the chromophore is believed to be the general mechanism for most RSFPs, structural studies reveal diversity in the details of photoswitching mechanisms, including different effects of protonation, chromophore planarity, and pocket flexibility. Applications of RSFPs include new types of live-cell superresolution imaging, tracking of protein movements and interactions, information storage, and optical control of protein activity.

Addresses
[1] Department of Bioengineering, Stanford University, Mailcode 5164, Stanford, CA 94305, United States
[2] Department of Pediatrics, Stanford University, Mailcode 5164, Stanford, CA 94305, United States

Corresponding author: Lin, Michael Z (mzlin@stanford.edu)

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In recent years considerable attention has been paid to phototransformable fluorescent proteins (FPs) because of their exciting new applications in superresolution fluorescence microscopy techniques [1,2]. Phototransformable FPs can be categorized into three types — photoactivating, photoconverting, and photoswitching — based on their responses to light. In contrast to photoactivation and photoconversion, which result from irreversible light-induced covalent modification of chromophore structures, photoswitching results from reversible conformational changes that allow the chromophore to switch between 'on' and 'off' states [3$^{\bullet\bullet}$]. Because of their ability to undergo repeated cycles of activation and deactivation, reversibly photoswitchable FPs have found unique utility in superresolution time-lapse microscopy in living cells. They have also been the subject of intense structural study to understand how alternate chromophore states exist and interconvert within a single protein. Finally, recent FP engineering efforts have succeeded in adjusting multiple performance parameters of photoswitchable FPs to improve their utility in biological experiments. This review will provide a summary of our understanding of photoswitchable FPs, describing recent findings on their basic switching mechanisms and summarizing their applications.

Basic characteristics of photoswitchable FPs

Several engineered mutants of the first FP cloned, the green fluorescent protein from *Aequoria victoria*, were known to exhibit switching properties in a portion of the protein population, such as YFP [4], CFP [5], EYFP [5], Citrine [5], E^{2}GFP [6], and YFP-10C [7]. However, these proteins generate limited contrast before and after light switching, preventing them from being widely utilized as photoswitchable highlighters. In 2003, the first efficiently photoswitchable FP, kindling fluorescent protein (KFP), was evolved from asFP595 and shown to be capable of precise *in vivo* photolabeling to track movements of proteins [8]. However, the tetrameric nature of asFP595 and its variants limited their practical use.

In the following year, Dronpa [9], a monomeric green photoswitchable FP, was engineered from a tetrameric Pectiniidae coral FP. Several mutants, PDM1-4 [10], Dronpa-2 [11], Dronpa-3 [11], rsFastLime [12], and bsDronpa [13], were evolved from Dronpa and show different photoswitching kinetics. These photoswitchable FPs show a baseline 'on' state that can be switched 'off' by light. Padron [13], another Dronpa mutant, is a photoswitchable FP that displays the opposite behavior of being 'off' at baseline and switching to 'on' upon illumination. In recent years, Mut2Q [14], EYQ1 [14], rsEGFP [15] and mGeos [16$^{\bullet}$] were reported to display different switching speed, faster maturation, better stability, or higher localization precision potential, serving as potential candidates to replace Dronpa in various biological applications. Furthermore, to expand the spectra window from GFPs, cyan-emitting mTFP1 [17] and several improved red photoswitchable FPs — rsCherry

[18], rsCherryRev [18], rsTagRFP [19] and mApple [20] — were also generated.

Two other types of engineered photoswitchable FPs are more complex in exhibiting other phototransforming properties in addition to photoswitching. One type comprises FPs that integrate both reversible photoswitching between on/off state and irreversible photoconversion from a green-emitting to a red-emitting form. This type includes IrisFPs [21,22] and NijiFP [23]. Their multiple phototranformation modes enable novel applications such as two-color nanoscopy and sequential photoactivation schemes. The second type is represented by a single YFP called Dreiklang [24•], which excites at 515 nm but switches at 405 and 365 nm. In most photoswitchable FPs, illumination at the wavelength for fluorescence excitation can also photoswitch the protein. Dreiklang is a unique photoswitchable FP in that its fluorescence excitation spectrum is decoupled from that for optical switching. This feature allows fine-tuning of the duration of the chromophore states without interference by the fluorescence excitation light. A summary of photoswitchable FP characteristics is presented in Table 1.

Mechanism of photoswitching

General mechanism: *cis–trans* isomerization

Photoswitchable FPs adopt a classic 11-strand beta-barrel FP structure that encloses an autocatalytically generated 4-(p-hydroxybenzylidene)-5-imidazolinone (p-HBI) chromophore. Structural studies of simple photoswitchable FPs indicate that *cis–trans* isomerization of the chromophore methylene bridge between the two rings of the chromophore can account for the photoswitching mechanism (Figure 1). In the cases that have been studied so far, for FPs that switch completely from on to off, the chromophore adopts the *cis* conformer in the resting state (Figure 1a), while FPs exhibiting off–on switching adopt the *trans* conformer at rest (Figure 1b). Stabilizing interactions between chromophore and the surrounding residues determine their resting states, for example, in Dronpa, the strong hydrogen bonding interaction between Ser142 and the hydroxybenzylidene moiety stabilizes its *cis* conformation, making Dronpa an on–off switch, while a single mutation Met159Tyr, as found in Padron, reverses the switching direction, because a hydrogen bond between Tyr159 and the p-hydroxyphenyl ring stabilizes the *trans* conformer of the chromophore.

The consistent association of *cis* and *trans* chromophore conformers with bright and dark states observed in all FPs characterized as photoswitching is not due to inherent properties of *cis* and *trans* chromophores. Indeed, there are FPs that exhibit brighter fluorescence in the *trans* than the *cis* conformation [25,26], and that transition between the two conformations upon illumination [27]. Thus these FPs could be considered as partial photoswitchable FPs
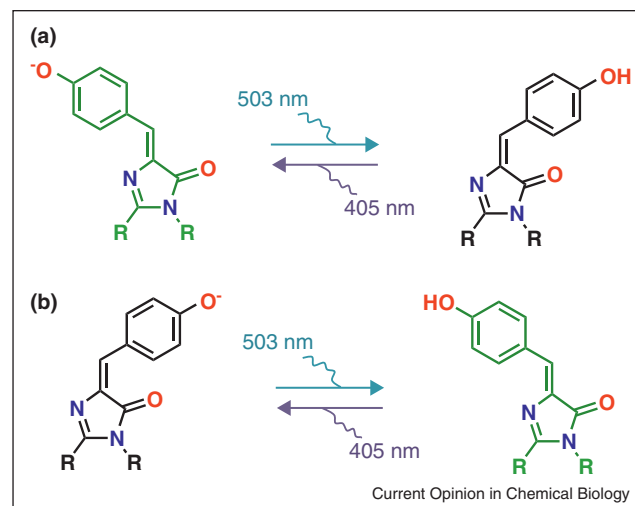
that operate in the opposite direction with respect to chromophore conformation. This emphasizes that attributes other than the chromophore conformer, such as modulation of absorbance spectra by chromophore protonation or modulation of quantum yield by chromophore flexibility, determine the relative brightness of the two conformers.

Chromophore protonation occurs in the off state of many photoswitchable FPs, leading to a blue-shift of the absorbance peak. This leads to a drop of absorption at the previous absorption wavelength and therefore an effective loss of fluorescence excitability. However, the blue-shifted protonated chromophore is also not fluorescent, so in these proteins additional differences in the flexibility of the chromophore in the bright and dark states must account for the dimming. Increases in chromophore torsion upon excitation, which have been predicted by molecular dynamics studies [28,29], are expected to decrease quantum yield regardless of spectral tuning. In Padron, these protonation-independent mechanisms appear to be the primary reason for the dimness of the basal state, as the basal *trans* chromophore is dim even when protonated. Furthermore, in Padron, a change in relative degree of protonation does not affect photoswitching [30,31]. Nevertheless, given the association of protonation with isomerization in most photoswitchable FPs, studies have addressed whether the two events are causally related with inconsistent results. In one study, isomerization was proposed to follow protonation [32], while in another, isomerization was believed to be the leading process [33]. Two other studies suggested a concerted process [14].

Photoswitching involves *cis–trans* isomerization in Dronpa **(a)** and *trans–cis* isomerization in Padron **(b)**.

Table 1

Well-characterized photoswitchable [FPs]

	Direction and Oligomerization	λ_{max} ex/em[a] (nm)	ε [a] (M^{-1} cm^{-1})	Φ[a]	Brightness relative to EGFP[a]	pK$_a$	$\lambda_{on/off}$ and $\lambda_{off/on}^a$ (nm/nm)
mTFP0.7 [17]	on–off (M[b])	453/488	60,000	0.50	0.89	4.0	458/405
Dronpa [9]	on–off (M)	503/517	94,100	0.67	1.88	5.3	488/405
PDM1-4 [10]	on–off (T[b])	503/517	ND[b]	ND	ND	ND	488/405
Dronpa-2 [11]	on–off (M)	489/515	56,000	0.28	0.47	ND	488/405
Dronpa-3 [11]	on–off (M)	489/515	58,000	0.33	0.57	ND	488/405
rsFastLime [12]	on–off (M)	496/518	39,094	0.77	0.89	ND	488/405
bsDronpa [13]	on–off (M)	460/504	45,000	0.50	0.67	ND	488/405
Padron [13]	off–on (M[c])	503 (396) /522	43,000	0.64	0.82	ND	405/488
Padron* [13]	off–on (M)	503 (395) /519	58,000	0.62	1.07	ND	405/488
Mut2Q [14]	on–off (M)	496/507	54,000	0.28	0.45	6.0	478/405
rsEGFP [15]	on–off (M)	493/510	47,000	0.36	0.50	6.5	488/405
mGeos-F [16]	on–off (M)	504/515	53,135	0.85	1.33	5	488/405
mGeos-M [16]	on–off (M)	503/514	51,609	0.85	1.29	4.5-5	488/405
mGeos-C [16]	on–off (M)	505/516	76,967	0.81	1.84	6	488/405
mGeos-S [16]	on–off (M)	501/512	64,602	0.76	1.44	5-5.5	488/405
mGeos-E [16]	on–off (M)	501/513	69,630	0.75	1.54	6-6.5	488/405
mGeos-L [16]	on–off (M)	501/513	53,448	0.72	1.13	5-5.5	488/405
EYQ1 [14]	on–off (M)	510/524	73,000	0.72	1.56	6.9	514/405
asFP595 [47]	off–on (T)	572/595	56,200	<0.001	<0.002	ND	450/569
KFP1 [8]	off–on (T)	590/600	59,000	0.07	0.12	ND	458/532
rsCherry [18]	off–on (M)	572/610	80,000	0.02	0.05	6.0	450/550
rsCherryRev [18]	on–off (M)	572/608	84,000	0.005	0.01	5.5	550/450
rsTagRFP [19]	on–off (M)	567/585	36,800	0.11	0.12	6.6	570/445
mApple [20]	on–off (M)	568/592	75,000	0.49	1.10	6.5	570/480
IrisFP [21]	on–off (T)	488/516	57,800	0.48	0.83	5.7	488
IrisFP [21]	on–off (T)	551/580	27,000	0.50	0.40	6.8	561/440
mIrisFP [22]	on–off (M)	486/516	74,000	0.60	1.32	5.7	488/405
mIrisFP [22]	on–off (M)	546/578	26,000	0.44	0.34	7.0	561/440
NijiFP [23]	on–off (M)	469/507	41,100	0.64	0.78	7.0	488/405
NijiFP [23]	on–off (M)	469/507	41,100	0.64	0.78	7.0	488/405
Dreiklang [24]	on–off (M)	511/529	83,000	0.41	1.01	7.2	405/365

[a] λ_{max} ex/em, maximum of excitation/emission spectrum; ε, molar extinction coefficient; Φ, fluorescence quantum yield; brightness is the product of quantum yield and molar extinction coefficient expressed of the EGFP brightness. $\lambda_{on/off}$ and $\lambda_{off/on}$, wavelengths required for efficient reversible transitions.
[b] M, monomer; T, tetramer; ND, not determined.
[c] 15% dimer at 4°C.

An alternative view: contribution from the beta barrel

In some on–off photoswitchable FPs, isomerization is accompanied by substantial conformational change of the chromophore pocket [17,21,34]. In these cases, side chains that sterically affect the isomerization process influence the switching capability and switching speed of a given FP. For example, in Dronpa, Val157 and Met159 hinder the isomerization of the chromophore. Accordingly, Dronpa-2 (Met159Thr) and Dronpa-3 (Val157Ile, Met159Ala) exhibit faster off-switching kinetics [11]. However, in the off–on photoswitching FP Padron, conformational rearrangements of the chromophore pocket are more subtle [30]. Indeed, Padron photoswitching is as efficient at 100 K, a temperature at which protein dynamical breathing is negligible, as at room temperature, implying that the chromophore pocket does not substantially hinder photoswitching [30].

Figure 2
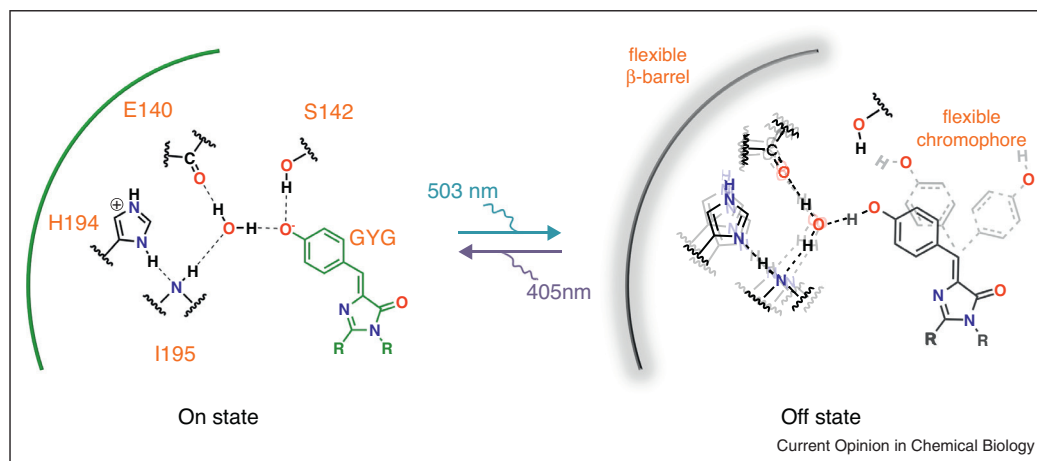
Conformational changes during Dronpa photoswitching at room temperature.

In an alternative view of Dronpa photoswitching, it was proposed that switching involves not only the formation of a *trans* conformer but also a dramatic increase in flexibility of the chromophore and the chromophore pocket (Figure 2). Mizuno *et al.* observed that a putative hydrogen-bond-donating serine residue located in the beta-barrel wall was required for a bright on-state, and that the wall of the beta-barrel structure near the chromophore becomes flexible in the off state, as detected by NMR [32]. The authors proposed that, instead of *cis–trans* isomerization driving protonation and an absorbance shift of the chromophore, protonation of the chromophore (through an unspecified process) first removes a hydrogen-bonding interaction with Ser142 in the beta-barrel wall, leading to local beta-barrel unfolding and then chromophore flexibility that lowers quantum yield.

However, the necessity of the beta barrel flexibility for loss of fluorescence was challenged by experiments showing that crystals in the off-state were as dim at ∼170 K as at room temperature [31]. If motion in the beta barrel were required for complete off-switching via quantum yield suppression, the off-state protein would be expected to be brighter at low temperatures, where motion is reduced, compared to room temperature, but this was not observed [31]. A mechanistic model that could account for all these observations could be that photoinduced *cis–trans* isomerization and loss of the hydrogen bond with Ser142 occurs together. At room temperature, this leads to beta-barrel disorder and then chromophore conformational flexibility, as was observed by NMR. The chromophore becomes protonated due to the loss of stabilization of the anionic state by the hydrogen bond from Ser142. At low temperatures, the beta

barrel may be essentially well ordered, and the chromophore may also be confined to a more restricted set of *trans* conformations. However, the chromophore could still become protonated from the loss of stabilization of the anionic state, and there may still be enough chromophore motion in the *trans* conformation to render it non-fluorescent. Regardless, some transient expansion or 'breathing' of the barrel may be required for off-switching, as viscosity in the surrounding environment [35•] and Dronpa oligomerization [10] result in slower kinetics of Dronpa off-photoswitching.

Different switching mechanism — reversible hydration/dehydration in Dreiklang

A unique photoswitchable FP, Dreiklang [24•], utilizes a completely different switching mechanism. Instead of *cis–trans* isomerization, the chromophore of Dreiklang undergoes a reversible hydration/dehydration reaction on a carbon atom in the imidazolinone ring (Figure 3). The hydration shortens the chromophoric π-electron system and makes the absorption wavelengths further blue-shifted. This new switching mechanism uniquely decouples the wavelengths used for photoswitching and for excitation for fluorescence detection in Dreiklang: peak wavelengths for reversible on-switching and off-switching are at ∼365 nm and ∼405 nm, whereas the fluorescence excitation spectrum peaks at ∼488 nm with emission peaking at ∼515 nm. Residues Y203, E222 and chromophore residue G65 were shown to be crucial for this reaction. A similar reversible hydration reaction was postulated to occur during the chromophore formation of GFP. We anticipate that with more engineering work, more photoswitchable FPs with decoupled switching and excitation wavelengths like Dreiklang could be generated, allowing for useful biological applications.

Figure 3
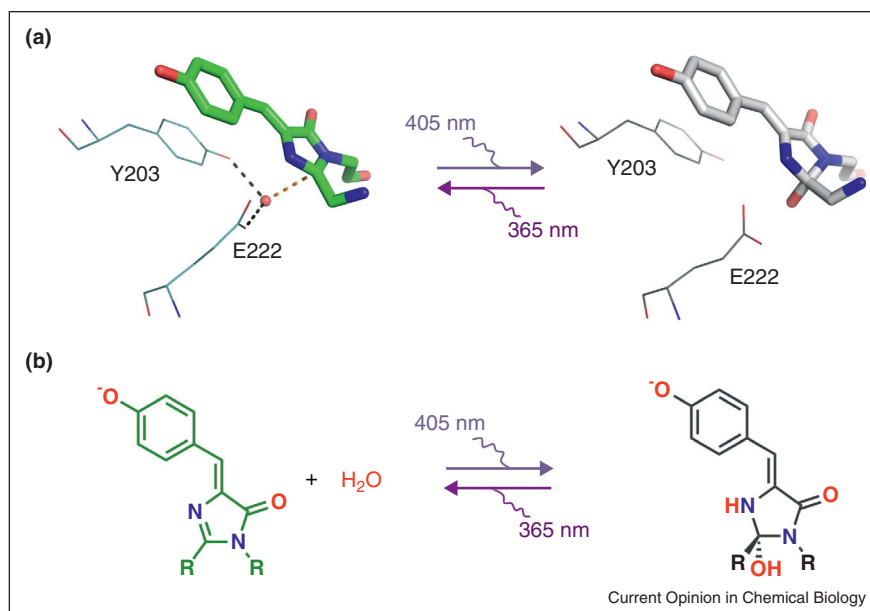
Conformational changes in Dreiklang photoswitching.

Applications

Tracking protein movement and interactions

Since their discovery, FPs have been extensively used to highlight protein of interest in living cells. However, it is difficult to track protein movement with non-transformable FPs since the labeled proteins would be evenly distributed in cells. Fluorescence recovery after photobleaching (FRAP) and optical activations of FPs are the two strategies to highlight select region of molecules and track their movements [36]. However, these methods are limited by their irreversible nature. Optical highlighting of photoswitching FPs enables the reversible labeling of specific molecules and thus enables the repeated measurements of protein behavior and the erasing of information after each measurement, thus allowing the identification of responses in one cell under different stimulus. Given these advantageous features, photoswitching FPs have been widely used for tracking protein dynamics in cells, for example, the observation of Erk translocation in and out of nucleus with and w/o EGF [9].

Another well known strategy using FPs is Förster resonance energy transfer (FRET), a popular technique to monitor protein interactions and conformational changes [37]. In this technique, FRET pair of cyan/yellow or green/red FPs are fused to two individual proteins to report their intermolecular interaction, or fused to one protein to flank its domain of interest and monitor its conformational change. Traditionally, photostable FPs would be preferable for FRET to guarantee reliable and consistent readouts. Recent years, with the report of the first red RSFP, rsTagRFP, photochromic FRET (pcFRET) method was proposed and demonstrated to show robust performance [19]. In this technique, the quantification of FRET efficiency is based on the measurements of donor fluorescence before and after light switching. Before photoswitching, there is a large overlap between donor emission and acceptor absorbance spectra, whereas after photoswitching, the donor emission and acceptor absorbance have small or no overlap. This internal change of the FRET pair allows accurate and repeated FRET quantification for the same FRET pair within the same live cell without the need for corrections based on reference images acquired from separate control cells.

Superresolution imaging

The observation of molecular events by traditional fluorescence imaging microscopy is hampered by the diffraction of light. Superresolution techniques can provide information about protein localization beyond the diffraction limit and thus can assist in elucidating protein functions and cell structures. Photoswitchable FPs are optimal fluorescent tags for superresolution imaging. It allows genetically labeling and repeatable data reading of target proteins. Here we briefly summarize the principles of three superresolution imaging techniques that use photoswitchable FPs as labels.

The first technique is patterned illumination-based superresolution, specifically reversible optically linear fluorescence transitions (RESOLFT) [15,38,39].

RESOLFT is evolved from stimulated emission depletion (STED) [40]. In RESOLFT, the protein of interest is labeled with photoswitchable FPs, and the sample is illuminated in a pattern that shapes like a doughnut and the intensity of light being small at one position. Only at this position, the molecules are not in the dark state and contribute to the detected signal. This region can be controlled to be smaller than the diffraction limit by increasing intensity of the transition light. The whole sample will be scanned to reconstruct the high-resolution image.

The second technique is single-molecule-based super-resolution reconstruction, specifically photoactivation-localization microscopy (PALM) and its variants [15,38]. This set of methods is based on sequential activation of fluorescent probes. During imaging, only a small number of molecules will be highlighted while the majority remain in the dark. The number of high-lighted molecules is optically resolvable in the sense that the imaged pixels can be interpreted as Gaussian distributions, and the pixel with the highest intensity would be located as the center of the corresponding molecule and form the 'located' molecule image. After each data collection, the fluorescent probes are subsequently deactivated and another subset of molecules is activated and imaged.

The third technique is photochromic stochastic optical fluctuation imaging (pcSOFI) [41•]. pcSOFI was evolved from stochastic optical fluctuation imaging using small chemical dyes (SOFI) [42]. In this method, an on-photoswitching FP is irradiated, which would produce robust single-molecule intensity fluctuations, from which a superresolution picture can be extracted by a statistical analysis of the fluctuations in each pixel as a function of time. Compared to the previous two methods, pcSOFI does not use specialized equipment and adopts simple and rapid data acquisition, serving as a widely accessible method for superresolution fluorescence imaging of living systems.

Sensor of subcellular environment and optical protein controller: applications based on beta-barrel flexibility

The occurrence of conformational changes in the side chains of beta-barrel residues forming the chromophore pocket during photoswitching implies that manipulations that increase flexibility of the beta-barrel could accelerate photoswitching. Indeed, the off-photoswitching speed of Dronpa and several of its variants decreases as the viscosity of the surrounding solvent increases, presumably because viscosity inhibits beta-barrel structural fluctuations required for photoswitching. Dronpa-3, a structurally more flexible mutant that exhibits robust viscosity dependence, was used as a genetically encoded microenvironment

Figure 4
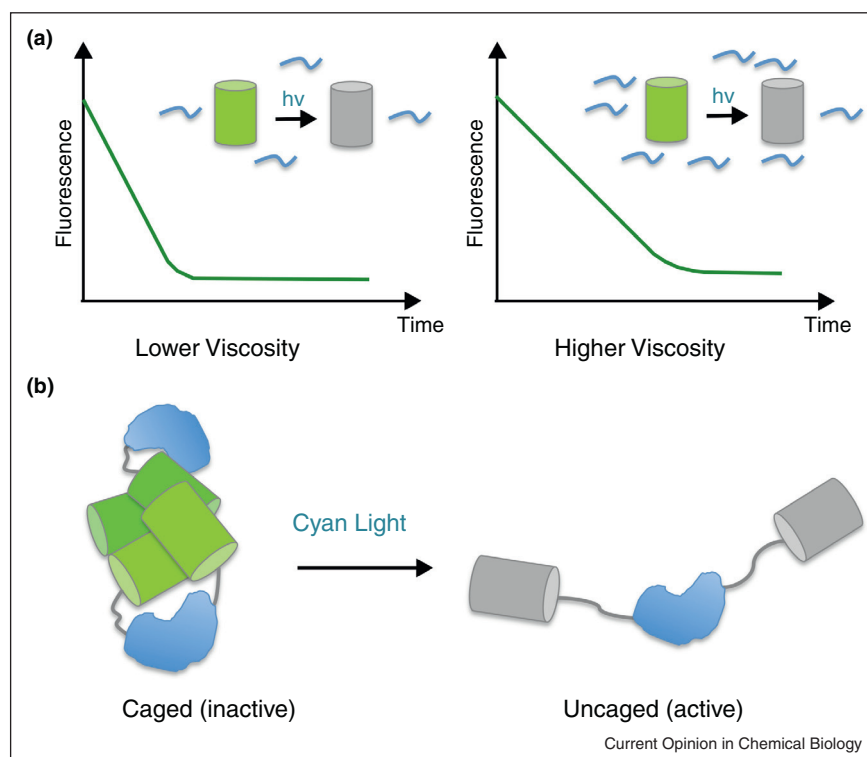
(a) Use of Dronpa-3 as a viscosity sensor. (b) A fluorescent light-inducible protein design based on Dronpa Lys145Asn.

probe to determine the differences in viscosities of different subcellular compartments [35[•]] (Figure 4a).

Another application is to develop a protein–activity actuator using Dronpa mutants [43[•]]. With off-photoswitching, beta strand 7 near the chromophore becomes flexible. This strand forms part of the cross-dimer interface in the tetrameric parent, and so it is reasonable to expect that off-photoswitching could affect the capability of Dronpa to oligomerize. Indeed, in the dark, Dronpa Lys145Asn is tetrameric, whereas cyan illumination induced redistribution from tetrameric toward monomeric species. On the basis of this light-dependent interaction, a fluorescent light-inducible protein (FLiPs) design was created, in which Dronpa Lys145Asn domain is fused to both termini of an enzyme of interest, where the termini straddle the enzyme active site. In the dark, the Dronpa Lys145Asn domains tetramerize and cage the protein, but light induces Dronpa Lys145Asn dissociation and activates the protein (Figure 4b). Thus Dronpa domains can function in reversible optical control of protein activities, a type of function which had previously been assumed to exist in only other types of chromophore-containing proteins. Conveniently, the photoswitchable fluorescence of Dronpa serves as a built-in read-out of the activity state of the target protein. It remains to be determined whether other photoswitchable FPs can also function as optical control elements.

Future applications in data writing and storage
A potentially useful application of photoswitchable FPs is optical data writing and storage. Unlike photoconvertible proteins, which can create red fluorescent patterns irreversibly created by light, photoswitchable FPs allow for multiple writing cycles [44]. 2D data writing has been performed with Dronpa and IrisFP coated on a surface, and 3D data writing in crystals of IrisFP and other EosFP mutants [27,45]. Compared to other optical encoding schemes such as encoding on silver zeolite microcarriers [46], photoswitchable FPs are not as stable, and physical separation is needed to create pixels or voxels. However, they may be of utility in situations where instability or biodegradability is desirable.

Summary
In the 10 years since the invention of KFP and Dronpa, photoswitchable FPs have found unique uses in the imaging of protein movements and in nanometer-scale precision localization of proteins. Just recently, a photoswitchable FP has been found to be capable of mediating control of protein activity with light, potentially expanding the uses of FPs from optical imaging to optical control. As a class of primarily artificial proteins, photoswitchable FPs continue to be the subject of protein engineering efforts as well as biophysical study to understand their unique structure and behavior. Without a doubt, the next decade will see more creative engineering and utilization of these capriciously colorful proteins.

Appendix A. Supplementary data
Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cbpa.2013.05.031.

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