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

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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.

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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••]. 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

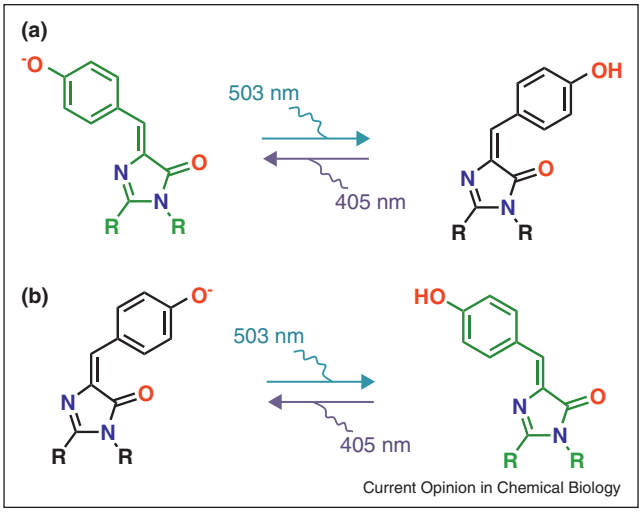
Several engineered mutants of the first FP cloned, the green fluorescent protein from *Aequoria victoria*, were known to exhibit switching properties in a portion of the protein population, such as YFP [4], CFP [5], EYFP [5], Citrine [5], E^2GFP [6], and YFP-10C [7]. However, these proteins generate 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•] 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.

Figure 1



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

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].