Review Article

Smart fluorescent proteins: Innovation for barrier-free superresolution imaging in living cells

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During the past decade, several novel fluorescence microscopy techniques have emerged that achieve incredible spatial and temporal resolution beyond the diffraction limit. These microscopy techniques depend on altered optical setups, unique fluorescent probes, or post-imaging analysis. Many of these techniques also depend strictly on the use of unique fluorescent proteins (FPs) with special photoswitching properties. These photo-switchable FPs are capable of switching between two states in response to light. All localization precision and patterned illumination techniques—such as photo-activation localization microscopy, stochastic optical reconstruction microscopy, reversible saturable optically linear transitions, and saturated structured illumination microscopy—take advantage of these inherent switching properties to achieve superior spatial resolution. This review provides extensive analysis of the positive and negative aspects of photoswitchable FPs, highlighting their application in diffraction-unlimited imaging and suggesting the most suitable fluorescent proteins for super-resolution imaging.

Key words: fluorescent protein, photoactivation localization microscopy, photoswitching, reversibly saturable optical fluorescence transition, stochastic optical reconstruction microscopy, superresolution imaging.

Introduction

Fluorescence bioimaging has become a revolutionary technique in many groundbreaking scientific technologies used to study biologically important parameters in living systems—for example, molecular dynamics, molecule interactions, signal transduction, biomolecule counting, biophysical and biochemical events (Donnert et al. 2007; Lippincott-schwartz & Patterson 2008; Lippincott-Schwartz & Manley 2009; Grotjohann et al. 2011; Wu et al. 2011; Shim et al. 2012). The most significant property of fluorescence bioimaging technology is its compatibility and ease of use with living systems. It provides easy, quick, and versatile molecular biological approaches. However, fluorescence microscopy techniques have intrinsic constraints in spatial resolution owing to the wave nature of light. In 1873, Ernest Abbe proposed a diffraction limit theory using light. His theory confirmed the resolution limit of fluorescence microscopy with the calculation \( \frac{\lambda}{2n\sin \alpha} \) in the focal plane (xy) and \( 2\lambda/n\sin^2 \alpha \) along the optical axis (z), where \( \lambda \) is the wavelength of the light, \( n \) is the refractive index of medium, \( \alpha \) is the half angle of the maximum cone of light entry or exit from objective lens, and \( n\sin \alpha \) is the numerical aperture of the objective lens. He suggested that spatial resolution could be improved only through the use of a shorter wavelength and a higher numerical aperture along with larger refractive index imaging medium. At that time, this theory was well acknowledged and thoroughly settled in scientific thinking. For decades, researchers sought strategies that could break the diffraction limit of optical microscopy.

Currently, Abbe’s diffraction limit theory is no longer a puzzle, and plenty of fluorescence microscopy techniques have achieved spatial resolutions of up to approximately 30–40 nm (far beyond Abbe’s diffraction limit of approximately 200 nm). These techniques are collectively known as superresolution microscopy. The scientific community is keen to achieve the goal of observing living cells on a molecular scale by extending superresolution fluorescence technology to scales of approximately 1 nm. In this review, we introduce superresolution microscopy techniques and the physicochemical properties of photoswitchable fluorescent proteins (PSFPs) and discuss their appropriate selection and application.
Superresolution microscopes

Many superresolution techniques are currently being used and can be roughly categorized in two groups based on underlying principles: (i) nonlinearity principle, and (ii) high-precision time-resolve localization of fluorophore. The first category comprises stimulated emission depletion (STED) microscopy (Hell et al. 1994; Klar et al. 2000; Hein et al. 2008), ground state depletion (GSD) microscopy (Hell & Kroug 1995; Fölling et al. 2008a; Manley et al. 2008), reversibly saturable optical fluorescence transition (RESOLFT) microscopy (Klar & Hell 1999), saturated structured illumination microscopy (SSIM) (Heintzmann et al. 2002), and bessel beam plane illumination microscopy (BBPLIM) (Planchon et al. 2011). Superresolution can be achieved with these microscopy techniques using conventional fluorophores, but recent approaches partly or fully depend on PSFPs to attain better spatial resolution (Fig. 1A,B). STED and GSD superresolution microscopy techniques are also utterly reliant on the use of a second laser (donut-shaped or overlapping depletion laser) after fluorophore excitation. This second laser maintains the diffraction-unlimited point spread function of fluorophores by depleting peripheral fluorescence. The major pitfall of these superresolution techniques is the use of powerful depletion or overlapping lasers, which are incompatible both with fluorophores and living objects; hence, these techniques have limitations in the extended imaging of living objects.

RESOLFT is an extension of STED principles based on photoswitchable fluorescent probes using standard far-field visible light optics with resolution well below the diffraction limit (approximately 30 nm) (Klar & Hell 1999; Grotjohann et al. 2011). In RESOLFT, superresolution is realized by altering the point spread function of ensemble photoswitchable fluorophores with low laser intensity. RESOLFT expeditiously detects emission from all fluorophores in nanosized objects. Several RESOLFT studies have been performed reconciled with PSFPs to attain resolution up to approximately 30 nm (Andresen et al. 2008; Brakemann et al. 2011; Grotjohann et al. 2011). SSIM is another nonlinearity principle-based microscopy. Conventional SSIM uses intense laser power to attain nonlinearity (Heintzmann et al. 2002; Gustafsson 2005), and extended SSIM technology now uses photoswitchable fluorophores to achieve nonlinearity by implementing very low laser power (Hofmann et al. 2005; Hirvonen et al. 2009; Rego et al. 2012). A recent study using SSIM accompanied by Dronpa (a reversible PSFP [R-PSFP]) has achieved up to approximately 50 nm resolution for nuclear pore imaging (Rego et al. 2012). Another recently developed microscopy, BBPLIM, is based on a thin light sheet structured illumination that achieves approximately 300 nm resolution in 3D with a conventional fluorophore (Planchon et al. 2011). We believe that the use of PSFPs in BBPLIM will further improve the resolution of this microscopy.

The second superresolution fluorescence imaging principle is based on the high-precision localization of a single fluorescence emitter. Diffraction-unlimited resolution can be achieved via localization precision microscopy techniques accompanied by special fluorophores using relatively low excitation power (Rust et al. 2006; Fernández-Suárez & Ting 2008). These special fluorophores can be either chemical dyes or genetically encoded fluorescent proteins (FPs) with inherent photoswitching properties. Using photoswitchable fluorescent probes in localization precision techniques excites and precisely localizes only a small
number of molecules in each frame. FPs are very useful in live-cell imaging because they can be expressed exogenously in cells alone or as fusion partners, making PSFPs widely applicable in superresolution techniques. Photoactivation localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM) are widely applicable superresolution microscopy techniques based on localization precision principles (see Fig. 1 and Table 1) (Gustafsson 2005; Hofmann et al. 2005; Hess et al. 2006; Huang et al. 2008). Various similar methods with different names have been invented—for example, fluorescence PALM, spectral precision distance measurement, direct STORM, PALM with independent running acquisition, and GSD and individual molecule return (Bornfleth et al. 1998; Heilemann et al. 2008; Egner et al. 2007; Fölling et al. 2008b). These superresolution techniques have been successfully implemented in several applications for diffraction-unlimited imaging of live cells, fixed biological samples, and multicolor imaging (Shroff et al. 2007; Gunkel et al. 2009; Subach et al. 2009; Huang et al. 2010; Lehmann et al. 2011; Bates et al. 2012; Lakadamyali et al. 2012). Although localization precision techniques achieve very high resolution, these methods center on the computationally aided localization of individual fluorophores, labeling density, and fluorophore selection.

**Advancements in fluorophores for superresolution imaging**

The application of green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* and its color variants as well as GFP-like proteins from other organisms has unquestionably revolutionized the capability to analyze a wide range of biological processes such as gene expression, protein localization, and cell motility in living specimens. However, this unique methodology has been enhanced by the development of PSFPs that make superresolution imaging practical and easy (Garini et al. 2005; Fernández-Suárez & Ting 2008; Day & Davidson 2009; Lippincott-Schwartz & Patterson 2009; Chudakov et al. 2010; Huang et al. 2010; Schermelleh et al. 2010; Brakemann et al. 2011; Grotjohann et al. 2011; Geissbuehler et al. 2011; Stepanenko et al. 2011; Subach et al. 2011a). The capability of PSFPs to change their spectral properties in response to irradiation with specific light has moved fluorescence imaging technology into a new and prodigious dimension. Various PSFPs have been invented and successfully implemented for diffraction-unlimited resolution in optical microscopy imaging. Among them are PSFPs that switch from a dark to a bright fluorescent state; others change fluorescence color from one emission state to another on switching. These proteins have significant impacts in superresolution fluorescence imaging. Commonly applicable and more efficient superresolution techniques such as PALM, STORM, SSIM, RESOLFT, stochastic optical fluctuation imaging (SOFI), and variance imaging for superresolution (Vision) are partly or fully dependent on the use of these special fluorophores (see Table 1) (Dertinger et al. 2009; Watanabe et al. 2010; Grotjohann et al. 2011; Rego et al. 2012; Zhu et al. 2012).

In general, PSFP’s switching time and brightness are the most important characteristics to consider for high-quality superresolution imaging. PSFPs undergo photoswitching during imaging; hence, the switching time between two states is crucial for collecting adequate frames before complete photobleaching (Grotjohann et al. 2011). Thus, the fast-switching FPs are preferential for collecting adequate frames with fast frame rate (Grotjohann et al. 2011). In addition, brightness and photostability are auxiliary stipulations for PALM/STORM imaging. The contrast ratio (or ratio of the on/off brightness) of PSFPs is also indispensable for better signal-to-noise ratio in high-quality superresolution imaging. This ratio varies largely among available PSFPs owing to differences in speed of switching between on/off states, high residual fluorescence (off-state fluorescence), and slow switching. Switching speed of R-PSFPs is largely varied among the existing FPs. The most acceptable explanation for switch on/off phenomena is based on cis-trans isomerization of chromophore, whereas the switching between these two states largely differs from one to another R-PSFP (Brakemann et al. 2010). Considering the above facts, PSFPs with high contrast ratios, fast switching, better photostability, and superior brightness are the best choices for superresolution imaging. In the case of reversible-PSFP (R-PSFP), high photostability is crucial for time-lapse imaging because a large number of images must be collected. By contrast, the use of irreversible PSFPs (Ir-PSFPs) requires a balance between the rate of photobleaching and that of photoactivation for localization precision-based superresolution imaging. Localized molecules must be bleached before others can be photoactivated. If the application is a cellular study, the FP should also be monomeric to minimize perturbation or misfolding of the target protein. Considering all characteristics, appropriate PSFP selection is tremendously important for attaining superior resolution.

**Properties of PSFPs**

As discussed above, PSFPs interchange between two states in response to defined light irradiation. Many
Table 1. Detail of superresolution microscopy techniques and summarized experimental conditions for imaging

<table>
<thead>
<tr>
<th>Superresolution techniques</th>
<th>Principle</th>
<th>Types of fluorescent protein</th>
<th>Resolution limit (X-axis) (nm)</th>
<th>Excitation power used(^a)</th>
<th>Live cell imaging(^b)</th>
<th>Use of PSFP(^c)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PALM</td>
<td>Wide field</td>
<td>R-PSFP, Ir-PSFP</td>
<td>20–30</td>
<td>**</td>
<td>***</td>
<td>*****</td>
<td>Betzig et al. (2006), Hess et al. (2006)</td>
</tr>
<tr>
<td>STORM</td>
<td>Wide field</td>
<td>R-PSFP, Ir-PSFP</td>
<td>20–30</td>
<td>**</td>
<td>***</td>
<td>****</td>
<td>Rust et al. (2006)</td>
</tr>
<tr>
<td>SIM</td>
<td>Wide field</td>
<td>Conventional</td>
<td>Approximately 100</td>
<td>***</td>
<td>***</td>
<td>*</td>
<td>Bailey et al. (1993), Gustafsson (2000)</td>
</tr>
<tr>
<td>SSIM</td>
<td>Wide field</td>
<td>Conventional</td>
<td>Approximately 50</td>
<td>**</td>
<td>****</td>
<td>****</td>
<td>Gustafsson (2005)</td>
</tr>
<tr>
<td>RESOLFT</td>
<td>Point scanning</td>
<td>R-PSFP, Ir-PSFP</td>
<td>Approximately 35</td>
<td>**</td>
<td>****</td>
<td>****</td>
<td>Hofmann et al. (2005), Klar &amp; Hell (1999)</td>
</tr>
<tr>
<td>4Pi</td>
<td>Point scanning</td>
<td>R-PSFP, Ir-PSFP</td>
<td>Approximately 100</td>
<td>****</td>
<td>***</td>
<td>**</td>
<td>Hell &amp; Wichmann (1994)</td>
</tr>
<tr>
<td>GSD</td>
<td>Point scanning</td>
<td>R-PSFP, Ir-PSFP</td>
<td>Approximately 100</td>
<td>*****</td>
<td>*</td>
<td>**</td>
<td>Hell &amp; Kroug (1995)</td>
</tr>
<tr>
<td>STED</td>
<td>Point scanning</td>
<td>Conventional</td>
<td>20–80</td>
<td>*****</td>
<td>*</td>
<td>*</td>
<td>Hell &amp; Wichmann (1994)</td>
</tr>
<tr>
<td>BBPLIM</td>
<td>Wide field</td>
<td>R-PSFP, Ir-PSFP</td>
<td>Approximately 200</td>
<td>*</td>
<td>****</td>
<td>****</td>
<td>Planchon et al. (2011)</td>
</tr>
<tr>
<td>SOFI</td>
<td>Wide field</td>
<td>R-PSFP, Ir-PSFP</td>
<td>Approximately 60</td>
<td>***</td>
<td>***</td>
<td>*****</td>
<td>Dertinger et al. (2009)</td>
</tr>
<tr>
<td>VIision</td>
<td>Wide field</td>
<td>R-PSFP, Ir-PSFP</td>
<td>Approximately 60</td>
<td>***</td>
<td>***</td>
<td>*****</td>
<td>Watanabe et al. (2010)</td>
</tr>
</tbody>
</table>

\(^a\) Indicates low or minimum value and (****) indicates high or maximum value. \(^b\) Requirement of excitation power is the major factor for live cell imaging. High excitation power is not biocompatible for living system and it caused photobleaching. We have shown a rough parameter of power uses, indicated by (*) lower to (****) higher. \(^c\) Compatibility of microscopy technique for live cell imaging is based on the light power required. Those techniques using lower power are more biocompatible for live cell imaging than the techniques needed very high power. Herein, (*) indicated least biocompatibility for live cell imaging and (****) indicated most biocompatible. \(^d\) This column is based on the recommendation of PSFP for superresolution microscopy techniques. Herein, (*) indicated that the techniques do not based on PSFP, while the (****) indicates the reliance of techniques on PSFP.
PSFPs have been developed and implemented for superresolution imaging (Lukyanov & Chudakov 2005; Shaner et al. 2007; Lippincott-schwartz & Patterson 2008; Nowotschin & Hadjantonakis 2009; Wu et al. 2011). Past research has shown that appropriate PSFP selection has a vital role in superresolution imaging; therefore, an elementary understanding of PSFPs is critical for their efficient selection in user-specific superresolution techniques. Owing to large differences in switching kinetics, fluorescence emission, excitation, photostability, maturation rate, and other characteristics, categorizing PSFPs into well-defined groups is difficult. As discussed above, the singular feature of a PSFP is its switching; thus, we group them into two categories based on their switching traits: (i) Ir-PSFPs, which irreversibly convert from one fluorescent state to another after irradiation (see Fig. 2), and (ii) R-PSFPs, which reversibly switch on and off with irradiation (see Fig. 2). Within these classes, PSFPs can be further subcategorized according to optical and biochemical characteristics and their applications in superresolution imaging.

**Ir-PSFPs**

Irreversible PSFPs irreversibly switch from one state to another in response to irradiation (see Fig. 2). Ir-PSFPs are ideal for the intracellular counting of proteins in live-cell and protein dynamics. R-PSFPs have disadvantages in this application owing to miscounting caused by reversible switching. Therefore, Ir-PSFPs show the highest potential in this field. Currently, several Ir-PSFPs and their improved versions have been developed and are available to provide solid options for diffraction-unlimited counting and imaging of living systems (Patterson & Lippincott-Schwartz 2002; McKinney et al. 2009; Subach et al. 2009; Baker et al. 2010; Annibale et al. 2011; Gunzenhäuser et al. 2012). We further categorize Ir-PSFPs into two groups: (i) those that photoswitch from a dark state to a bright fluorescent state or photoactivatable (PA) fluorescent proteins (PAFPs; Table 2), and (ii) those that photoconvert from one fluorescence to another, or photoconvertible fluorescent proteins (PCFPs; Table 3). Some examples of Ir-PSFPs include PSmOrange, PAmCherry, PAtagRFP, PACFP, PACFP2, Kae, PA-GFP, mEosFP, mEos2, mKikGR, Dendra, and Dendra2 (see Tables 2,3).

**PAFPs.** Photoactivatable fluorescent proteins are a subcategory of Ir-PSFPs. These FPs convert from a dark equilibrium state to bright fluorescent state after irradiation with distinct light (Figs 2, 3A). PA-GFP, PAmRFP1, PAmCherry1, PAmCherry2, and PAmCherry3 are currently used PAFPs from this group (see Table 2). PA-GFP was the first PSFP developed from wild-type GFP. PA-GFP can be photoactivated (emission max 520 nm) from its dark equilibrium state by ultraviolet (UV) irradiation. The superior properties of PA-GFP (e.g. brightness, contrast ratio, photostability, and fusion protein compatibility) have made them popular for diffraction-limited and diffraction-unlimited fluorescence imaging of living cells. They have been used successfully to demonstrate protein diffusion across the nuclear envelope, inter-lysosomal membrane protein exchange, temporal and spatial protein dynamics, single-cell tracking, and neuronal protein dynamics and for developmental studies of chick embryos.

![Fig. 2. Schematic of the switching pattern of photoswitchable fluorescent proteins (PSFPs). PSFPs are divided into two groups based on their switching behavior in response to irradiation. Each individual circle corresponds to single fluorophore. (A) R-PSFPs show reversible switching between on and off states upon light irradiation. Cyan circles represent switched on state while the black circle represents dark or switched off state of R-PSFP. (B) Irreversible (Ir)-PSFP shows switching from inactive to active state only once (herein, the inactive state is the initial state of the fluorescent protein [FP]), which comprise photoactivatable (PA)FP and photoconvertible (PC)FP. PAFP of this category exists in dark, non-fluorescent forms, which has switching ability from dark to bright fluorescent states after irradiation. The cyan circle represents the switched on state while the black circle represents the dark or switched off state. PCFP show photoconversion from initial fluorescent state to another fluorescent state in response to light irradiation. Cyan circle in PCFP represent initial fluorescent state while post-photoconversion state represent by red circle.](image-url)
PAmCherry1 has several outstanding features for both dual- and triple-color superresolution imaging with other PSFPs (Subach et al. 2009). After photoactivation, PAmRFP1 (emission max 605, quantum yield [QY] 0.08) demonstrates a 70-fold increase in fluorescence intensity. However, it has limited use in superresolution imaging owing to its low contrast ratio and extremely low quantum yield. PAmCherry1 has several outstanding features—for example, high QY (0.46), superior contrast ratio, excellent photostability, fast photoactivation, fast maturation, more suitable pH stability, and monomeric nature—that make it ideal for superresolution imaging (Subach et al. 2009). PAmCherry1 has been successfully used for both dual- and triple-color superresolution imaging of thick samples in two- and three-dimensional (3D) imaging (Izeddin et al. 2011; Zanacchi et al. 2011; Wilmes et al. 2012). PATagRFP is another bright red fluorescent Ir-PSFP (extinction coefficient 66 000/mol per cm, QY 0.38) (Subach et al. 2010a). Its monomeric nature under physiological conditions, high brightness, high contrast ratio, and superior single-molecule brightness in living cells make this FP an excellent choice for diffraction-limited and superresolution dual-color imaging with other PSFPs (Subach et al. 2010a).

Table 2. Details of irreversible photoswitchable fluorescent proteins (Ir-PSFPs) or photoactivatable fluorescent proteins (PAFPs) that switch from dark states to bright fluorescent state

<table>
<thead>
<tr>
<th>Ir-PSFP/Characteristics</th>
<th>PAmCherry1</th>
<th>PAmCherry2</th>
<th>PAmCherry3</th>
<th>PAGFP</th>
<th>KFP1</th>
<th>PAmRFP1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex (max), nm</td>
<td>564</td>
<td>570</td>
<td>570</td>
<td>504</td>
<td>580</td>
<td>578</td>
</tr>
<tr>
<td>Er (max), nm</td>
<td>595</td>
<td>596</td>
<td>596</td>
<td>517</td>
<td>600</td>
<td>605</td>
</tr>
<tr>
<td>Extinction coefficient</td>
<td>6500, 18 000</td>
<td>1900, 24 000</td>
<td>6500; 21 000</td>
<td>ND</td>
<td>59 000</td>
<td>10 000</td>
</tr>
<tr>
<td>Oligomeric state</td>
<td>Monomer</td>
<td>Monomer</td>
<td>Monomer</td>
<td>Monomer</td>
<td>Tetramer</td>
<td>Monomer</td>
</tr>
<tr>
<td>Chromophore switching</td>
<td>Irreversible</td>
<td>Irreversible</td>
<td>Irreversible</td>
<td>Irreversible</td>
<td>Irreversible</td>
<td>Irreversible</td>
</tr>
<tr>
<td>Switching light (nm)</td>
<td>Violet</td>
<td>Violet</td>
<td>Violet</td>
<td>ND</td>
<td>0.07</td>
<td>0.08</td>
</tr>
<tr>
<td>QY</td>
<td>0.46</td>
<td>0.53</td>
<td>0.24</td>
<td>ND</td>
<td>Dark</td>
<td>Dark</td>
</tr>
<tr>
<td>Initial color</td>
<td>Dark</td>
<td>Dark</td>
<td>Dark</td>
<td>Dark</td>
<td>Green</td>
<td>Red</td>
</tr>
<tr>
<td>Color after activation</td>
<td>Red</td>
<td>Red</td>
<td>Red</td>
<td>Red</td>
<td>PALM,</td>
<td>STORM</td>
</tr>
<tr>
<td>Application in superresolution techniques</td>
<td>PALMIRA, STORM</td>
<td>PALMIRA, SPALM, STORM</td>
<td>PALMIRA, SPALM, STORM</td>
<td>PALM, STORM</td>
<td>STED, SIM, PALM, STORM</td>
<td></td>
</tr>
<tr>
<td>Source Reference</td>
<td>mCherry</td>
<td>mCherry</td>
<td>mCherry</td>
<td>GFP</td>
<td>asCP</td>
<td>mRFP</td>
</tr>
</tbody>
</table>

In addition to PA-GFP, PA-mRFP1 and PAmCherry1 have been developed from DsRed. These FPs emit red fluorescence after photoactivation with intense violet light (Verkhusha & Sorkin 2005; Subach et al. 2009). After photoactivation, PAmRFP1 (emission max 605, quantum yield [QY] 0.08) demonstrates a 70-fold increase in fluorescence intensity. However, it has limited use in superresolution imaging owing to its low contrast ratio and extremely low quantum yield. PAmCherry1 has several outstanding features—for example, high QY (0.46), superior contrast ratio, excellent photostability, fast photoactivation, fast maturation, more suitable pH stability, and monomeric nature—that make it ideal for superresolution imaging (Subach et al. 2009). PAmCherry1 has been successfully used for both dual- and triple-color superresolution imaging of thick samples in two- and three-dimensional (3D) imaging (Izeddin et al. 2011; Zanacchi et al. 2011; Wilmes et al. 2012). PATagRFP is another bright red fluorescent Ir-PSFP (extinction coefficient 66 000/mol per cm, QY 0.38) (Subach et al. 2010a). Its monomeric nature under physiological conditions, high brightness, high contrast ratio, and superior single-molecule brightness in living cells make this FP an excellent choice for diffraction-limited and superresolution dual-color imaging with other PSFPs (Subach et al. 2010a).

PCFPs. As another subclass of Ir-PSFPs, PCFPs exist in a bright equilibrium state and have the capability to photoconvert from one emission state to another in response to irradiation at specific wavelengths (see Figs 2, 3B). Among these, Kaede, KikGR, mKikGR, ClavGR2, Dendra, Dendra2, PSCFP, PSCFP2, EcoFP, mEos2, mEcos3, PSmOrange, and PSmOrange2 are the most recently developed Ir-PSFPs (see Table 3). Kaede is an Ir-PSFP originating from the story coral Trachyphyllia geoffroyi (Ando et al. 2002). After irradiation with UV light (350–400 nm), Kaede undergoes irreversible photoconversion from green to red fluorescence with QYs of 0.80 and 0.33, respectively. Kaede has shown potential in individual neuron imaging in neuron-dense environments (Ando et al. 2002). The considerable drawback of Kaede is its tetrameric structure, which is unsuitable as a fusion protein tag in live-cell imaging and superresolution microscopy imaging. Another engineered protein, KikGR, was developed more recently from a non-photoconvertible protein (Tsutsui et al. 2005). KikGR also exists in tetrameric form and is therefore ill suited for fusion tagging in cellular studies. In 2008, a monomeric version of KikGR was developed that maintains similar brightness (QY 0.69 and 0.63 before and after activation, respectively). Owing to its monomeric nature and superior brightness, mKikGR is applicable to live-cell and single-molecule imaging in precision localization techniques (Habuchi et al. 2008).

Dendra is a tetrameric Ir-PSFP derived from Octocoral dendronephthya (Gurskaya et al. 2006). Dendra and its subsequent versions are the only Ir-PSFPs that use blue light (480 nm) for photoconversion. Dendra2 is an improved version of Dendra developed through single amino acid substitution (A224V). Dendra2 is superior to Dendra in maturation and brightness in both the pre- and the post-photoswitching states (http://www.evrogen.com/protein-
<table>
<thead>
<tr>
<th>Protein</th>
<th>Ex (max), nm</th>
<th>Em (max), nm</th>
<th>Extinction coefficient (mL/mol per cm)</th>
<th>Oligomeric state</th>
<th>Chromophore switching</th>
<th>Switching light</th>
<th>QY</th>
<th>Initial color</th>
<th>Color after activation</th>
<th>Super-resolution Imaging application</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSmOrange</td>
<td>548, 638</td>
<td>565, 651</td>
<td>113, 320</td>
<td>Monomer</td>
<td>Irreversible</td>
<td>Blue-Green</td>
<td>0.51</td>
<td>Orange</td>
<td>Red</td>
<td>In vivo imaging, PALM, STORM</td>
<td>Subach et al. (2011a)</td>
</tr>
<tr>
<td>PSmOrange2</td>
<td>546, 619</td>
<td>51, 000</td>
<td>47, 900</td>
<td>Monomer</td>
<td>Irreversible</td>
<td>Blue-Green</td>
<td>0.61</td>
<td>Red</td>
<td>Red</td>
<td>In vivo imaging, PALM, STORM</td>
<td>Subach et al. (2012)</td>
</tr>
<tr>
<td>Phamret</td>
<td>458, 490</td>
<td>43, 000</td>
<td>ND</td>
<td>Monomer</td>
<td>Irreversible</td>
<td>UV-violet</td>
<td>0.59</td>
<td>Cyan</td>
<td>Green</td>
<td>Optical highlighter, PALM, STORM</td>
<td>Matsuda et al. (2008)</td>
</tr>
<tr>
<td>PSCFP-2</td>
<td>400, 490</td>
<td>47, 000</td>
<td>ND</td>
<td>Monomer</td>
<td>Irreversible</td>
<td>UV-violet</td>
<td>0.69</td>
<td>Cyan</td>
<td>Green</td>
<td>Optical highlighter, PALM, STORM</td>
<td>McKinney et al. (2009)</td>
</tr>
<tr>
<td>Kaede</td>
<td>507, 573</td>
<td>507, 573</td>
<td>103, 18</td>
<td>Tetramer</td>
<td>Irreversible</td>
<td>UV-Violet</td>
<td>0.64</td>
<td>Green</td>
<td>Red</td>
<td>Optical highlighter, PALM, STORM</td>
<td>Habuchi et al. (2012)</td>
</tr>
<tr>
<td>mKikGR</td>
<td>519, 584</td>
<td>67, 200</td>
<td>37, 000</td>
<td>Monomer</td>
<td>Irreversible</td>
<td>UV-Violet</td>
<td>0.54</td>
<td>Green</td>
<td>Red</td>
<td>Optical highlighter, PALM, STORM</td>
<td>Habuchi et al. (2009)</td>
</tr>
<tr>
<td>Dendra2</td>
<td>56, 000</td>
<td>56, 000</td>
<td>ND</td>
<td>Monomer</td>
<td>Irreversible</td>
<td>UV-Violet</td>
<td>0.84</td>
<td>Red</td>
<td>Red</td>
<td>Optical highlighter, PALM, STORM</td>
<td>Zhang et al. (2012)</td>
</tr>
<tr>
<td>EosFP</td>
<td>490, 553</td>
<td>500, 570</td>
<td>29, 000</td>
<td>Monomer</td>
<td>Irreversible</td>
<td>UV-Violet</td>
<td>0.83</td>
<td>Orange</td>
<td>Red</td>
<td>PALM, STORM, RESOLFT, PALM, STORM</td>
<td>McKinney et al. (2009)</td>
</tr>
<tr>
<td>mEos2</td>
<td>519, 584</td>
<td>56, 000</td>
<td>ND</td>
<td>Monomer</td>
<td>Irreversible</td>
<td>UV-Violet</td>
<td>0.83</td>
<td>Red</td>
<td>Red</td>
<td>PALM, STORM, RESOLFT, PALM, STORM</td>
<td>Habuchi et al. (2009)</td>
</tr>
<tr>
<td>mEos3</td>
<td>519, 584</td>
<td>68, 400</td>
<td>ND</td>
<td>Monomer</td>
<td>Irreversible</td>
<td>UV-Violet</td>
<td>0.83</td>
<td>Red</td>
<td>Red</td>
<td>PALM, STORM, RESOLFT, PALM, STORM</td>
<td>Habuchi et al. (2009)</td>
</tr>
</tbody>
</table>

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photoconvert from green to red fluorescence state to a red fluorescence state upon UV-violet light stimulation. Dendra2 is capable of irreversible photoconversion from green to red fluorescence in response to blue light irradiation. Dendra2 outperforms its counterpart in a variety of factor-limited and superresolution studies—for example, vesicle tracking in neuronal gap junctions (Falk et al. 2009), protein tracking using fluorescence imaging with 1-nm accuracy (i.e. FIONA) in Caenorhabditis elegans (Kural et al. 2009), neuronal branching studies (Flynn et al. 2009), and multi-compartment protein trafficking studies in live cells (Chudakov et al. 2007a).

Whereas most existing Ir-PSFPs photoconvert from green to red emission states, PSCFP and PSCFP2 are the only available Ir-PSFPs that convert from cyan to green (Chudakov et al. 2004). PSCFP emits 468-nm cyan fluorescence in response to excitation with 400 nm, whereas intense 400-nm light causes photoconversion of PSCFP (emission max 511 nm and excitation max 490 nm). PSCFP2 is an improved version of PSCFP, and both were developed by the same researchers (Chudakov et al. 2007a). Compared to its predecessor, PSCFP2 has the advantages of faster maturation and brighter fluorescence both before (QY 0.2) and after (QY 0.23) photoconversion. Its superior contrast ratio (approximately 400 times) is an additional benefit that improves signal-to-noise ratio in superresolution imaging (Chudakov et al. 2004). PSCFP2 also has advantages in superresolution imaging because its pre- and post-conversion excitation (excitation max 468 nm and 511 nm, respectively) and emission spectra (emission max 400 nm versus 490 nm, respectively) are completely decoupled from each other. PSCFP2 is useful alone as well as for dual-color and single-molecule imaging with other PSFPs (Shroff et al. 2007; Zhang et al. 2007).

EosFP and its variant mEosFP stand out owing to their brightness, which is superior to that of all other existing Ir-PSFPs. This characteristic makes these FPs widely applicable for superresolution imaging (Wiedenmann et al. 2004; Wiedenmann and Nienhaus 2006; McKinney et al. 2009). In its equilibrium state, EosFP exhibits green fluorescence (excitation max 505 nm and emission max 516 nm). EosFP can be photoconverted from a green to a red emission state (excitation max 561 and emission max 581 nm) after excitation at 380 nm. Wild-type EosFP is unsuitable for fusion tagging in live-cell imaging owing to its tetrameric nature. The monomeric version (mEosFP) has almost all of the properties of EosFP with a slightly lower QY (0.64) and extinction coefficient (67 200/mol per cm). mEosFP has been successfully implemented in frogs to study early embryo morphogenesis (Wacker et al. 2007). The main disadvantage of mEosFP is chromophore formation only below 30°C, which limits its use for animal cell studies. However, mEos2 (an improved variant of mEosFP) has better folding efficiency, superior spectral properties, high brightness, fast photoconversion, high contrast ratio, and maturation properties, making it ideal for fusion tagging in live-cell imaging at 37°C. mEos2 is an excellent PSFP candidate for several factor-limited and superresolution studies—for example, protein counting within a factor-limited region (Lee et al. 2012), live-cell superresolution imaging of yeast (Young et al. 2012), cell tracking, and protein dynamics (Baker et al. 2010). Very recently, Zhang et al. (2012) have shown that mEos2 has oligomer characteristics at high concentration and aggregates in membrane-localized live cells. They also engineered mEos3.1 and mEos3.2 (improved variants of mEos2) to have truly monomeric natures, high brightness, high photon budget, and fast maturation. These variants will likely supersede mEos2 in the future.

Phamret is a unique photoconvertible protein composed of monomeric super enhanced CFP (msecFP) and PA-GFP. It is the only available Ir-PSFP that can be excited by single laser (458 nm) for both the pre- and post-photoconverted states (Matsuda et al. 2008). Phamret can be photoconverted from cyan fluorescence (emission max 480 nm) to green fluorescence (emission max 520 nm) after UV irradiation with an approximately 12 times higher brightness contrast ratio. This characteristic makes Phamret a perfect FP for the visualization of protein dynamics and diffusion patterns (Fig. 4) (Matsuda et al. 2008). Phamret is

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extraordinarily useful for researchers who cannot afford multiple laser sources.

The recently developed PSmOrange is a robust example of an Ir-PSFP that is initially orange (excitation max 548 nm and emission max 565 nm) but switches to far-red (excitation max 636 nm and emission max 662 nm) after irradiation with blue-green light. Compared to all other existing PSFPs, PSmOrange has most far-red excitation spectra with greater brightness, faster maturation, higher photoconversion contrast, and better photostability (Subach et al. 2011a). These characteristics are central to superresolution imaging, and the red-shifted spectrum of PSmOrange enables its simultaneous use for dual-color imaging with green PSFPs. Because PSmOrange behaves as a true monomer, it is suitable for fusion tagging in live-cell imaging. PSmOrange is also useful for both in vitro and in vivo imaging owing to its far-red emission, superior brightness, and monomeric behavior under physiological conditions (Subach et al. 2011b). Very recently, an improved variant, PSmOrange2, was developed that displays nearly four times faster switching kinetics and slightly blue-shifted photoconversion fluorescence compared with that of PSmOrange (Subach et al. 2012). These changes will likely lead to PSmOrange2 being substituted for PSmOrange in the future.

R-PSFPs

In brief, R-PSFPs can be repeatedly switched between dark and bright fluorescence states, respectively, with light irradiation (see Figs 2, 5, and Table 4). R-PSFPs are the most useful FPs for not only localization precision-based superresolution techniques (e.g. PALM, STORM) but also wide-field superresolution techniques (e.g. SSIM and BBPLIM) and point scanning superresolution techniques (e.g. RESOLFT). R-PSFP proteins can be further subdivided into two groups—positive switching and negative switching—based on the light used for on/off switching and fluorophore excitation. Many R-PSFPs have been developed: for example, Dronpa, rsFastLime, rsCherryRev, rsCherry, broad-spectrum Dronpa (bsDronpa), Padron, rsEGFP, etc.
Table 4. Details of reversible photoswitchable fluorescent proteins (R-PSFPs), which switch reversibly between two states

<table>
<thead>
<tr>
<th>Protein</th>
<th>Ex (max), nm</th>
<th>Em (max), nm</th>
<th>Extinction coefficient (mol per cm)</th>
<th>Oligomeric state</th>
<th>Switching pattern</th>
<th>Equilibrium state</th>
<th>Equilibrium (%fluorescence)</th>
<th>Residual fluorescence in off state (%)</th>
<th>Bleaching/cycle (%)</th>
<th>Switch On</th>
<th>Switch off</th>
<th>Color change</th>
<th>QY (ON)</th>
<th>Chromophore</th>
<th>Switching mechanism</th>
<th>Relaxation half time (min)</th>
<th>Application in superresolution techniques</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>mGeos-X</td>
<td>501–506</td>
<td>493</td>
<td>515</td>
<td>Monomer</td>
<td>Reversible negative</td>
<td>ON</td>
<td>ND</td>
<td>1.90–6.42</td>
<td>2.56–11.52</td>
<td>405</td>
<td>405</td>
<td>Dark/Green</td>
<td>0.72–0.85</td>
<td>ND</td>
<td>ND</td>
<td>169–398</td>
<td>STORM, PALM, RESOLFT, EGFP</td>
<td>Chang et al. (2012)</td>
<td></td>
</tr>
<tr>
<td>rsEGFP</td>
<td>512–519</td>
<td>510</td>
<td>529</td>
<td>Monomer</td>
<td>Reversible negative</td>
<td>ON</td>
<td>ND</td>
<td>1.40</td>
<td>ND</td>
<td>491</td>
<td>405</td>
<td>Dark/Green</td>
<td>0.36</td>
<td>ND</td>
<td>ND</td>
<td>23</td>
<td>STORM, PALM, RESOLFT, EGFP</td>
<td>Grotjohann et al. (2011)</td>
<td></td>
</tr>
<tr>
<td>Dreiklang</td>
<td>47 000</td>
<td>83 000</td>
<td>43 000</td>
<td>Monomer</td>
<td>Reversible positive</td>
<td>OFF</td>
<td>ND</td>
<td>0.70</td>
<td>ND</td>
<td>405</td>
<td>405</td>
<td>Dark/Green</td>
<td>0.41</td>
<td>Hydration-dehydration</td>
<td>ND</td>
<td>150</td>
<td>STORM, PALM, RESOLFT, EGFP</td>
<td>Stiel et al. (2011)</td>
<td></td>
</tr>
<tr>
<td>Padron</td>
<td></td>
<td></td>
<td></td>
<td>Monomer</td>
<td>Reversible positive</td>
<td>OFF</td>
<td>ND</td>
<td>6</td>
<td>2</td>
<td>488</td>
<td>405</td>
<td>Dark/Green</td>
<td>0.64</td>
<td>Hydration-dehydration</td>
<td>150</td>
<td>54</td>
<td>RESOLFT, STORM, SSIM</td>
<td>Andresen et al. (2008)</td>
<td></td>
</tr>
<tr>
<td>Padron</td>
<td></td>
<td></td>
<td></td>
<td>Monomer</td>
<td>Reversible negative</td>
<td>ON</td>
<td>5</td>
<td>0.50</td>
<td>50.5</td>
<td>405</td>
<td>405</td>
<td>Dark/Green</td>
<td>0.64</td>
<td>Hydration-dehydration</td>
<td>5</td>
<td>405</td>
<td>RESOLFT, STORM, SSIM</td>
<td>Andresen et al. (2008)</td>
<td></td>
</tr>
<tr>
<td>bsDronpa</td>
<td></td>
<td></td>
<td></td>
<td>Monomer</td>
<td>Reversible negative</td>
<td>OFF</td>
<td>95</td>
<td>0.5</td>
<td>17</td>
<td>488</td>
<td>488</td>
<td>Dark/Green</td>
<td>0.5</td>
<td>cis-trans conversion</td>
<td>93</td>
<td>100</td>
<td>PALM, STORM, SSIM</td>
<td>Andresen et al. (2008)</td>
<td></td>
</tr>
<tr>
<td>rsFastLime</td>
<td></td>
<td></td>
<td></td>
<td>Monomer</td>
<td>Reversible negative</td>
<td>OFF</td>
<td>93</td>
<td>0.6</td>
<td>17</td>
<td>488</td>
<td>488</td>
<td>Dark/Green</td>
<td>0.6</td>
<td>cis-trans conversion</td>
<td>100</td>
<td>100</td>
<td>PALM, STORM, SSIM</td>
<td>Andresen et al. (2008)</td>
<td></td>
</tr>
<tr>
<td>Dronpa</td>
<td></td>
<td></td>
<td></td>
<td>Monomer</td>
<td>Reversible negative</td>
<td>ON</td>
<td>100</td>
<td>0.6</td>
<td>17</td>
<td>488</td>
<td>488</td>
<td>Dark/Green</td>
<td>0.6</td>
<td>cis-trans conversion</td>
<td>100</td>
<td>100</td>
<td>PALM, STORM, SSIM</td>
<td>Andresen et al. (2008)</td>
<td></td>
</tr>
<tr>
<td>Dronpa-2</td>
<td></td>
<td></td>
<td></td>
<td>Monomer</td>
<td>Reversible negative</td>
<td>ON</td>
<td>100</td>
<td>0.6</td>
<td>17</td>
<td>488</td>
<td>488</td>
<td>Dark/Green</td>
<td>0.6</td>
<td>cis-trans conversion</td>
<td>ND</td>
<td>100</td>
<td>PALM, STORM, SSIM</td>
<td>Andresen et al. (2008)</td>
<td></td>
</tr>
<tr>
<td>Dronpa-3</td>
<td></td>
<td></td>
<td></td>
<td>Monomer</td>
<td>Reversible negative</td>
<td>OFF</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Dark/Green</td>
<td>0.6</td>
<td>cis-trans conversion</td>
<td>ND</td>
<td>ND</td>
<td>ND, not determined.</td>
<td>Andresen et al. (2008)</td>
<td></td>
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<tr>
<td>rsCherry</td>
<td></td>
<td></td>
<td></td>
<td>Monomer</td>
<td>Reversible negative</td>
<td>ON</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Dark/Green</td>
<td>0.6</td>
<td>cis-trans conversion</td>
<td>ND</td>
<td>ND</td>
<td>ND, not determined.</td>
<td>Andresen et al. (2008)</td>
<td></td>
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<tr>
<td>rsCherryRev</td>
<td></td>
<td></td>
<td></td>
<td>Monomer</td>
<td>Reversible negative</td>
<td>OFF</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Dark/Green</td>
<td>0.6</td>
<td>cis-trans conversion</td>
<td>ND</td>
<td>ND</td>
<td>ND, not determined.</td>
<td>Andresen et al. (2008)</td>
<td></td>
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</tbody>
</table>

ND, not determined.
Dreiklang, and mGeosX (see Table 4). We discuss the positive and negative aspects of R-PSFPs in superresolution imaging below.

**Negative-switching R-PSFP.** Negative-switching R-PSFPs use similar wavelengths for switching off and fluorophore excitation (see Fig. 5A). Hence, these FPs are ill suited for time-lapse imaging. Dronpa was the first monomeric R-PSFP engineered through directed and random mutagenesis (Ando et al. 2004). It can be repeatedly switched between dark to bright states or vice versa with 405 nm and 488 nm irradiation, respectively. The switching mechanism of Dronpa is explained by light-driven cis-trans chromophore isomerization (Stiel et al. 2007). The bright equilibrium state of Dronpa has an exceptionally high QY (0.85) and extinction coefficient (125 000/mol per cm), which makes it capable of imaging and protein dynamics studies in living cells (e.g. nuclear import and export of mitogen-activated protein kinase, in vivo single-neuron imaging, stress activity, and membrane ruffling) and dual-color 3D superresolution imaging using photochromic stochastic optical fluctuation imaging (Fig. 6) (Ando et al. 2004; Fujioka et al. 2006; Aramaki & Hatta 2006; Kurokawa & Matsuda 2005; Lummer et al. 2011; Dedecker et al. 2012). Despite the wide applications of Dronpa, its weak photostability, long photoswitching time, and negative photoswitching properties limit its use in living systems. These drawbacks spurred improvements in Dronpa for superresolution imaging, leading to the development of Dronpa-2, Dronpa-3, and rsFastLime. These improved variants of Dronpa display rapid photoswitching and are useful for diffraction-unlimited live-cell imaging and protein dynamics (Ando et al. 2007; Stiel et al. 2007; Kao et al. 2012). In 2008, Andresen et al. developed another fast-switching Dronpa variant called bsDronpa that has a high contrast ratio and a slightly lower QY (0.50) and extinction coefficient (45 000/mol per cm). It has the advantages of a broad absorption spectra and large Stork shift (absorbance max 460 nm and emission max 504 nm), which make it applicable for dual-color imaging with Padron because its switching behavior is opposite and its emission spectra largely overlaps those of Padron (Andresen et al. 2008).

A turning point in R-PSFP technology was the development of rsEGFP (Grotjohann et al. 2011). rsEGFP is a fast-maturing, monomeric green fluorescent protein developed from EGFP by introducing a crucial Q69L mutation with four additional mutations. rsEGFP exists in a bright fluorescent equilibrium state (QY 0.36 and extinction coefficient 47 000/mol per cm). Similar to Dronpa, rsEGFP can be repeatedly switched on and off at 405 nm and 491 nm, respectively, and it can produce 120 times the number of switching cycles of Dronpa. rsEGFP is currently considered the best choice in all aspects for superresolution imaging given its advanced properties, including fast switching speed, large number of switching cycles, high photo-stability (Fig. 7) (Grotjohann et al. 2011). However, Dronpa remains superior to rsEGFP in terms of both QY and extinction coefficient, and therefore it is still the preferred choice for superresolution imaging.

![Fig. 6. Superresolution photochromic stochastic optical fluctuation imaging (pcSOFI) of HeLa cells. (A, B) Three-dimensional imaging of HeLa cells labeled with DAKAP-Dronpa. Images A and B depict conventional and pcSOFI images, respectively. (C, D) Dual-color pcSOFI imaging of HeLa cells labeled with Lyn-Dronpa (green) and Kras-rsTagRFP (red). Images C and D show the conventional and pcSOFI images, respectively. The scale bar is 10 μm in all images (Dedecker et al. 2012).](image-url)
exceptionally useful for diffraction-unlimited studies (Kao et al. 2012).

Another recently developed negative-switching R-PSFP, mGeosX, displays fast switching, pH resistance, and high brightness (Chang et al. 2012). mGeosX FPs were developed from mEos2 through site-directed random mutagenesis at the first amino acid of its chromophore. mGeos mutants differ from one another only at the first position of the tripeptide (XYG) of their chromophores and exhibit varied photoswitching rates, photon budgets, photostability, and acid sensitivities. The palettes of mGeosX provide the freedom to choose the most suitable FP according to experimental requirements. The switching kinetics of

Fig. 7. (A) Performance of rsEGFP for rewritable data storage and coding into binary language (Grotjohann et al. 2011). rsEGFP embedded in PAA was used to read 17 × 17 μm area of the text of 25 Grimm’s fairy story (ASCII code; 1.9Mbits). The graph represents intensity profile of the marked region and the blue line indicates the threshold used 0 or 1 for spot. (B) Reversible saturable optically linear transitions (RESOLFT) imaging of Dreiklang in living animal cells. Superresolution microscopy of living PtK2 cells expressing Keratin19-Dreiklang, Images were taken in the confocal (left) and RESOLFT (center) modes. Images on the right side show magnified regions marked in the images on the left. Scale bars in the left and middle images are 1 μm; those in right images are 250 nm (Brakemann et al. 2011 and Grotjohann et al. 2011).
mGeosX are similar to those of Dronpa and can be turned on and off with 405-nm and 488-nm lasers, respectively. These R-PSFPs have a maximum absorption of 501 nm to 505 nm, an emission max of 512 nm to 519 nm, and a very high QY (0.72–0.85). Almost all properties of mGeosX are nearly identical to those of other Dronpa variants; therefore, it adds versatility to the current toolkit of PSFPs for superresolution imaging.

Nearly all of the R-PSFPs discussed thus far emit green fluorescence. rsCherryRev and rsCherry are red-emission R-PSFPs developed from mCherry that maintain negative and positive switching, respectively (Stiel et al. 2008). rsCherryRev is considerably superior to its positive-switching variant rsCherry in terms of dynamic range and contrast ratio. The main drawback with these proteins is the relatively high residual fluorescence in the off state compared with that of other existing R-PSFPs. Thus, the superresolution imaging application of rsCherryRev is limited even though its single-molecule brightness and red emission makes it a potential partner with green PSFPs. rsTagRFP is another red-emitting R-PSFP that is relatively better than rsCherryRev. rsTagRFP switches under blue and yellow light irradiation into a red fluorescent state on and a dark off state, respectively. Again, rsTagRFP has limited application in superresolution imaging owing to its low QY and high background signal (Saubach et al. 2010b; Pletnev et al. 2012). Owing to the limited color variants of R-PSFPs, this field has exceptional opportunity for expansion with new color variants.

Positive-switching R-PSFP. For positive-switching R-PSFPs, similar or nearly identical wavelengths are used for switching on and fluorophore excitation (see Fig. 5B). Positive switching R-PSFPs are very useful for 3D superresolution imaging owing to the accessibility of similar wavelengths for excitation and switching on. rsCherry is a monomeric red-emitting R-PSFP (Stiel et al. 2008). In its on state, rsCherry absorbs at 572 nm and emits at 610 nm. It can be turned on and off by 550 nm and 450 nm, respectively. Despite its positive switching, rsCherry is not widely applicable in superresolution imaging owing to low QY and high residual signal in the off state (15%).

Andresen et al. (2008) have also developed another improved positive-switching Dronpa variant known as Padron. Padron has attracted significant attention from the scientific community because of its positive-switching behavior and other advantages such as fast switching, high brightness for imaging, robust contrast, and fast maturation rate. Padron was developed by directed error-prone mutagenesis alternating with site-directed saturation mutagenesis at amino acids with close proximity to the chromophore. In contrast to Dronpa, Padron can be turned off at 405 nm and on to excitation at 488 nm. Padron exists in dark equilibrium state and is turned on with exposure to cyan light irradiation (extinction coefficient, 43 000/mol per cm, QY, 0.64, and emission max 522 nm). Similar to that of Dronpa, the on/off switching in Padron occurs through cis-trans chromophore isomerization (Brakemann et al. 2010). Padron is considered better than Dronpa because of its positive-switching behavior, which is useful for extended imaging. Padron has been used for dual-color imaging with the Dronpa variant bsDronpa (Andresen et al. 2008) even though they have nearly overlapping emission spectra.

Dreiklang is another excellent contribution to the R-PSFP toolkit. Dreiklang is a monomeric GFP variant that can be switched on and off by 365 nm and 405 nm light, respectively (Brakemann et al. 2011). A third laser (515 nm) is also needed for fluorophore excitation. Dreiklang displays a bright fluorescent equilibrium state (QY 0.41 and extinction coefficient 83 000/mol per cm). Padron is better than Dreiklang owing to its high QY (0.64) but the extinction coefficient (43 000/mol per cm), which is nearly half that of the latter. Given its monomeric nature, Dreiklang is an ideal candidate for fusion tagging in live-cell imaging and achieves up to approximately 35-nm resolution (see Fig. 7) (Brakemann et al. 2011). The only drawback with Dreiklang is the requirement for three laser lines in the microscopy system. A complete switching cycle for Dreiklang requires excess UV light, which always creates difficulty for live-cell imaging. Compared to negative-switching rsEGFP (approximately 1200 cycles), Dreiklang produces just one-tenth of the switching cycles, but its positive-switching characteristics are useful for time-lapse imaging superresolution imaging. On the other hand, the negative switching R-PSFP uses similar wavelength for both excitation and switched off, thus the R-PSFPs partially turned off with the laser required for fluorophore excitation. To maintain the signal/noise ratio using negatively switching R-PSFP, higher excitation power is required due to the gradual turned off of fluorophore, which always causes photobleaching. While in positive switching R-PSFP, excitation light and switch on light work in co-operative manner due to the similarity in wavelength, which kept all fluorophore turned on during acquisition process. rsEGFP and Dreiklang, both have homologies similar to that of GFP; therefore, we strongly advocate the improvement of the switching cycle of Dreiklang.

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Concluding remarks

Currently, several monomeric PSFPs have emerged, and the process of their improvement is ongoing. As we discuss herein, superresolution techniques such as PALM, STORM, RESOLFT, and SSIM are capable of achieving 20–40 nm resolutions because of their special features. The selection of suitable fluorophores largely determines the achievable resolution of superresolution microscopy techniques. In the present scenario, the PSFP toolkit is limited primarily to red or green fluorescence emission. Only a few PSFPs—for example, Phamret and PCFP2—emit colors outside of the green or red emission range (Chudakov et al. 2007a; Matsuda et al. 2008). Therefore, the development of photo-switchable fluorescent probes with new colors and superior photoswitching properties is urgently needed. PSFPs with various color choices will facilitate multicolor superresolution microscopy. Recently, a fast-switching rsEGFP was developed that can rewrite and store data with fast imaging. rsEGFP produces an exceptional number of switching events. Nonetheless, color variants with rsEGFP for multicolor imaging that can produce a large number of switching events remains unavailable (Grotjohann et al. 2011). Conversely, positive-switching R-PSFPs such as Padron and Dreiklang are useful for 3D superresolution imaging, but their superficial performance (Low number of switching cycles and moderately slow switch on/off) limits their efficient use in superresolution techniques (Andresen et al. 2008; Brakemann et al. 2011). Further enhancements of PSFPs will certainly improve imaging technology, but present available resources remain adequate for the exploration of many hidden biological events.

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References


