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Highlights of the optical highlighter fluorescent proteins

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Summary

The development of super-resolution microscopy techniques using molecular localization, such as Photoactivated Localization Microscopy (PALM), Fluorescence Photoactivated Localization Microscopy (F-PALM), Stochastic Optical Reconstruction Microscopy (STORM), PALM with Independent Running Acquisition (PALMIRA) and many others, has heightened interest in molecules that will be grouped here into a category referred to as “optical highlighter” fluorescent proteins. This review will survey many of the advances in fluorescent protein for optically highlighting molecule subsets within a population of fluorescently labeled molecules.

Keywords

photoactivation; photoconversion; photoswitching; fluorescence microscopy

Introduction

The term “optical highlighter” describes molecules that are initially non-fluorescent or can be made non-fluorescent at the activated fluorescence wavelength and increase in fluorescence with contrast over a darker background after photoactivation, photoconversion, or photoswitching. In addition to super-resolution techniques (Patterson, 2009), these offer an alternative to photobleaching approaches in the study of protein kinetics, gene expression, organelle dynamics, and even cellular dynamics within living specimen. Numerous advances and discoveries have been made in developing optical highlighter fluorescent proteins, of which only a subset is discussed here. These molecules have been classified in several manners. Here, a selection of fluorescent proteins is generally grouped into three subclasses: photoactivatable (off state to on state), photoconvertible (converts from one wavelength to another), or photoswitchable (switches on and off repeatedly). Readers should note that synthetic optical highlighter probe development has progressed much over recent several years. And although these have been developed or modified for many of the same uses, discussion is limited here to their fluorescent protein counterparts.

Fluorescent proteins: Green photoactivation

The wild type GFP (wtGFP) from *Aequorea victoria* was the first GFP used as highlighter. The chromophore population is a mixture of neutral phenols (Y66 is protonated) and anionic phenolates (Y66 is deprotonated) which produce absorption peaks at ~397 nm and ~475 nm, respectively. The technique relied on the photoconversion taking place after irradiation with ~400 nm light (Yokoe & Meyer, 1996), which produces an absorption increase at the minor peak and gives a subsequent increase in the fluorescence intensity when excited at this wavelength. This approach was improved using mutants at the T203 position (Patterson & Lippincott-Schwartz, 2002). A T203I mutant produces a variant that reduces the minor absorbance peak while maintaining the major peak (Heim *et al.*, 1994, Ehrig *et al.*, 1995).

Several substitutions at this position, including T203I, were found to undergo photoconversion (Patterson & Lippincott-Schwartz, 2002). The T203H variant (named PA-GFP) decreased 488 nm absorption (Fig 1A) and produced >60 fold fluorescence (Fig. 1B) increases after ~400 nm irradiation (Patterson & Lippincott-Schwartz, 2002). (Table 1)

Fluorescent proteins: Cyan-to-green photoactivation

Photoswitchable cyan fluorescent protein (PS-CFP) changes both excitation and emission spectra in a shift from a cyan to a green fluorescent protein after irradiation (Chudakov *et al.*, 2004) and could perhaps be considered photoconvertible, but is thought to have a structural alteration similar to wtGFP and PAGFP when photoactivated (Lukyanov *et al.*, 2005). PS-CFP initially displays excitation at 402 nm and emission at 468 nm (Chudakov *et al.*, 2004). (Fig 1C) After activation with ~400 nm light, new excitation and emission peaks are observed at 490 nm and 511 nm, respectively (Fig. 1D) producing ~1500-fold increase in the green-to-cyan fluorescence ratio. An improved version, PS-CFP2, that is brighter and develops fluorescence more efficiently at 37°C is also available through the commercial source, Evrogen.

Fluorescent proteins: Red photoactivation

The monomeric version of DsRed, mRFP1 (Campbell *et al.*, 2002), was made into a series of photoactivatable fluorescent proteins, PAmRFP1-1, PAmRFP1-2, and PAmRFP1-3 (Verkhusha & Sorkin, 2005). The brightest, PAmRFP1-1, has a quantum yield of only 0.08, but produces an ~70-fold increase in red fluorescence upon ultraviolet light irradiation (Verkhusha & Sorkin, 2005). This was expanded by development of mCherry (a derivative of mRFP1) into a photoactivatable marker. PAmCherry1 (Subach *et al.*, 2009) also has little fluorescence before photoactivation but was found to display a fluorescence increase of ~4000 fold and be ~10 times brighter than PAmRFP1-1 after ~400 nm irradiation. (Fig. 1E and 1F) The brightness of the TagRFP (Merzlyak *et al.*, 2007) made it an appealing target for developing a photoactivatable fluorescent protein. Indeed, PATagRFP (Subach *et al.*, 2010) is ~3 times brighter than PAmCherry1 with an extinction coefficient and a quantum yield of 66,000 M^{-1} cm^{-1} and 0.38, respectively. Both PAmCherry1 (Subach *et al.*, 2009) and PATagRFP (Subach *et al.*, 2010) have been used successfully in diffraction-limited photolabeling and super-resolution PALM experiments.

Fluorescent proteins: Green-to-red photoconversion

Kaede was one of the first green-to-red photoconvertible proteins utilized in mammalian cell biology (Ando *et al.*, 2002). Before photoconversion with ~400 nm light, it has a major absorbance peak at 508 nm and emission at 518 nm. (Table 1) After photoconversion, it has a new red-shifted absorbance peak at 572 nm, which upon excitation fluoresces with a new emission peak at 582 nm. This shift in both the excitation and emission peaks results in a >2000 fold increase in the red-to-green fluorescence ratio. Kaede forms tetramers (Ando *et al.*, 2002), which is a limitation for some experiments, but its large contrast over background after photoconversion makes it appealing as a marker for cells in developing organisms.

Another green-to-red photocovertible fluorescent protein (Tsutsui *et al.*, 2005) was developed from KikG (*Favia favus*) which did not originally have highlighting properties. Using structural characterizations of Kaede, KikG was engineered into the KikGR (Fig. 1G and 1H), which produced >2000-fold increase in fluorescence contrast during ratio imaging of the red and green components. (Table 1) The purified KikGR protein lacked the brightness of Kaede, but displayed several fold more fluorescence than Kaede when expressed in cells. It was also found to be a tetramer (Tsutsui *et al.*, 2005), but has since

been modified into the monomeric version, mKikGR (Habuchi *et al.*, 2008), which expands the use of this highlighter in cell biology applications.

EosFP also exhibits a green-to-red fluorescence photoconversion upon ultraviolet or near ultraviolet light irradiation (Wiedenmann *et al.*, 2004). It has a green excitation maximum at 506 nm with emission at 516 nm until irradiation at 405 nm, which produces an excitation peak located at 571 nm with emission at 581 nm. (Table 1) Initially determined to be a tetramer, EosFP was engineered into two dimeric forms, d1EosFP and d2EosFP, and into a monomeric mEosFP. The emission maxima of these mutants remain constant while excitation maxima and brightness change slightly (Table 1). The mEosFP inefficiently forms a fluorescent molecule when expressed at 37°C (Wiedenmann *et al.*, 2004), so an alternative for using the better performing dimeric forms and avoiding the problems associated with unintended intermolecular dimerization of tagged proteins has been to link two of them together into a tandem dimer, tdEosFP. Since having twice the size can also result in problems with use, the monomeric version was further developed into an improved protein, mEos2 (McKinney *et al.*, 2009). The mEos2 is one of the brightest optical highlighter fluorescent proteins, has good photostability, and provides localization precisions in PALM imaging on the order of ~10 nm.

Dendra gives a 4500-fold photoconversion from its green-to-red fluorescent forms (Gurskaya *et al.*, 2006). (Table 1) The wild type dendGFP (Labas *et al.*, 2002) was engineered into the monomeric Dendra with its photoconversion properties. An improved version, Dendra2, represents an improvement in folding efficiency at 37°C. Differing from most of the proteins discussed here, Dendra and Dendra2 offer the options of being photoconverted with ~400 nm light or with a potentially less phototoxic wavelength (~488 nm).

Fluorescent proteins: Photoswitchable

One of the first reported proteins which could be efficiently switched on and off in bulk measurements was Dronpa. It initially displays green fluorescence with an absorption maximum at 503 nm (Fig. 1I) and emission maximum of 518 nm (Fig. 1J) (Ando *et al.*, 2004). With intense irradiation at 490 nm, the absorbance at 503nm as well as the green fluorescence emission is decreased. However, after irradiation at 400 nm, the 503 nm absorbance as well as the green fluorescence emission is rapidly restored. (Table 1) Remarkably, Dronpa can undergo the on-off cycling >100 times with a loss of only 25% of the original fluorescence. The reversible nature of the fluorescence allows the same photoactivation experiment to be repeated multiple times within the same region of interest (Ando *et al.*, 2004). This molecule has also undergone numerous alterations to produce several variants. Dronpa-2 and Dronpa-3 (Ando *et al.*, 2007) were found to photoswitch off much more efficiently than the original. The rsFastlime mutant was found to photoswitch in both directions more efficiently (Stiel *et al.*, 2007). The bsDronpa variant (Andresen *et al.*, 2008) has a broad absorption spectrum which is blue-shifted compared with Dronpa and the other variants. And Padron (Andresen *et al.*, 2008) shows photoswitching under opposite irradiation protocols by being turned on by 488 nm and off by 405 nm irradiation (Andresen *et al.*, 2008). In addition to protein tracking, Dronpa and some of its derivatives, rsFastlime and Padron, have also shown their utility in super-resolution molecular localization experiments (Andresen *et al.*, 2008).

Other photoswitchable fluorescent proteins include two versions of mCherry, rsCherry and rsCherryRev (Stiel *et al.*, 2008). (Table 1) These molecules are switched back and forth between on and off states using red and green/yellow light. The rsCherry behaves similarly to Padron in that the more red-shifted irradiation (561 nm) turns it on while the more blue-

shifted irradiation (470 nm) turns it off. IrisFP, a derivative of EosFP discussed earlier, can be irreversibly photoconverted just as EosFP, but has also joined the photoswitchable category by having the capability to photoswitch between off and on states while in the unconverted green form as well as photoswitch between on and off red states after photoconversion (Adam *et al.*, 2008).

Fluorescent proteins: Conventional proteins behaving unconventionally

Included in this section are the fluorescent proteins that are normally just expressed and imaged without any irradiation step to increase their fluorescence. The conventional fluorescent proteins, EGFP (Haupts *et al.*, 1998), EYFP (Dickson *et al.*, 1997, Schwille *et al.*, 2000, Biteen *et al.*, 2008), Citrine (Heikal *et al.*, 2000), and PhiYFP (Folling *et al.*, 2008), have all been found to sample dark or other fluorescent states during single molecule imaging and Fluorescence Correlation Spectroscopy (FCS). In addition, several of the widely used red and orange variants have also displayed photoconversion into species fluorescing at wavelengths other than their original (Kremers *et al.*, 2009). Of the variants studied, Katusha, mKate, HcRed1, mPlum, and mRaspberry had blue shifts of their spectra to varying degrees, whereas mOrange1, mOrange2, and Kusabira-Orange displayed red spectral shifts after irradiation. While these suggest caution in using these molecules in photobleaching studies, the authors pointedly showed that these molecules can be used in highlighting experiments also (Kremers *et al.*, 2009).

Summary and outlook

The key feature of optical highlighter molecules is that they provide the capability to contrast a sub-population of tagged molecules over the entire pool within a specimen. These sub-populations can be monitored temporally and spatially using conventional diffraction-limited imaging techniques (several applications reviewed in (Lippincott-Schwartz & Patterson, 2009)) or for several super-resolution imaging techniques (Patterson, 2009). Based on publications over the past few years, the development of fluorescent protein optical highlighters has for the most part followed that of conventional fluorescent proteins and the goals will likely continue to be to make them better and redder. For instance, users of just about any fluorescence technique have better molecular brightness of the probes as the feature at the top of their wish list. This is especially key for the optical highlighter fluorescent proteins since they often lack the brightness of their conventional fluorescent protein counterpart. (Table 1) Shifting the excitation and emission wavelengths toward the infrared part of the spectrum is another goal that is passed along from conventional fluorescent protein development. However, for optical highlighters requiring irradiation in the near UV wavelength spectrum, improvements to red-shift the photoactivation, photoconversion, or photoswitching wavelengths are additional goals.

Will the renewed interest in optical highlighters continue to drive the probe development and eventually evolve these techniques into commonly used approaches for biologists? The super-resolution techniques relying on these probes have produced a great deal of excitement from biologists whom wish only to observe their favorite subject with a little more clarity. In addition, commercial microscope companies have been implementing “photoactivation” modules into their systems for several years and some are even producing commercial versions of instruments and software used for (F)PALM and STORM. This combination of investigator and commercial interests will likely drive the field and make common the use of optical highlighters over the next few years.

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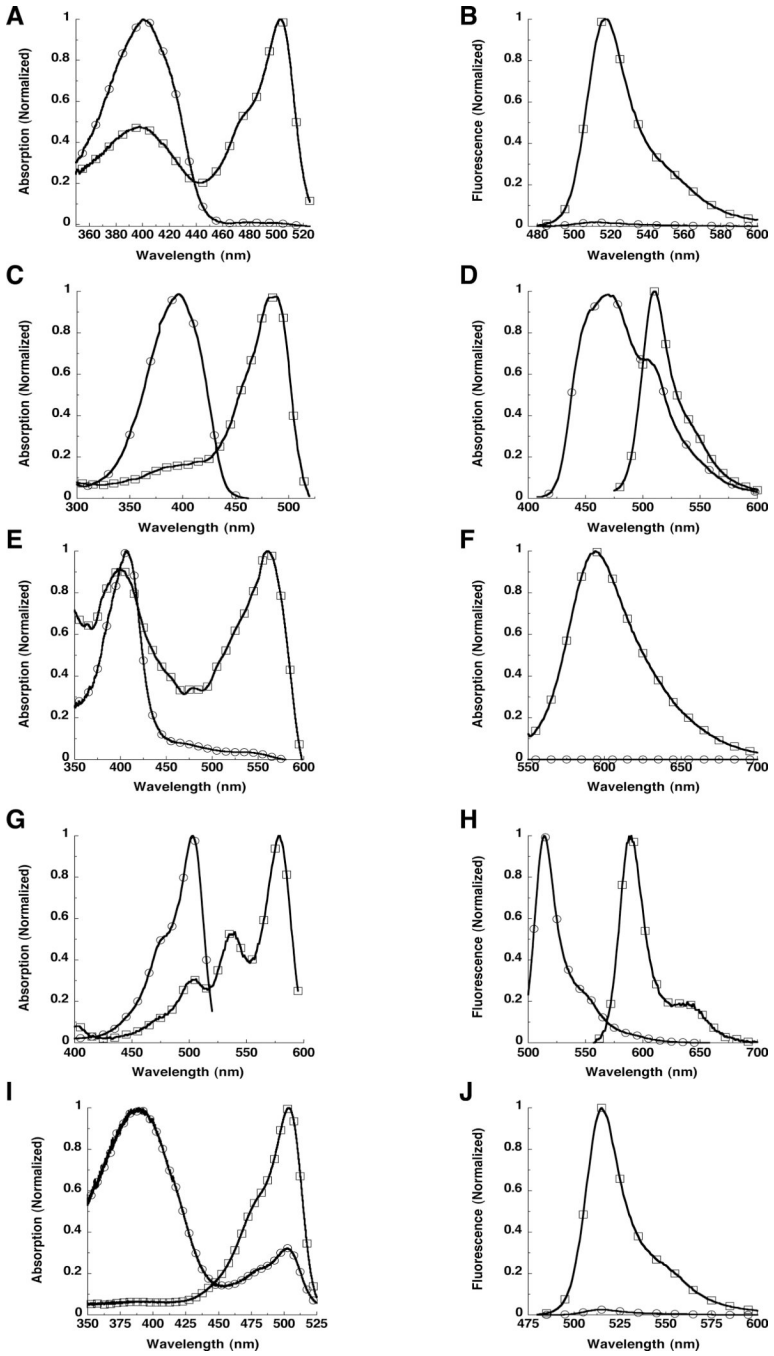

Figure 1. Example spectra for several optical highlighter fluorescent proteins

A. Absorption spectra of PAGFP before (open circles) and after (open squares) photoactivation. **B.** Emission spectra of PAGFP before (open circles) and after (open squares) photoactivation. **C.** Absorption spectra of PSCFP before (open circles) and after (open squares) photoactivation. **D.** Emission spectra of PSCFP before (open circles) and after (open squares) photoactivation. **E.** Absorption spectra of PAmCherry1 before (open circles) and after (open squares) photoactivation. **F.** Emission spectra of PAmCherry1 before (open circles) and after (open squares) photoactivation. **G.** Absorption spectra of mKiKGR before (open circles) and after (open squares) photoconversion. **H.** Emission spectra of mKiKGR before (open circles) and after (open squares) photoconversion. **I.** Absorption

spectra of Dronpa in the OFF state (open circles) and after photoswitching to the ON state (open squares). **J.** Emission spectra of Dronpa in the OFF state (open circles) and after photoswitching to the ON state (open squares). Note that the Dronpa protein sample was not entirely switched to the dark state (open circles) in this example.

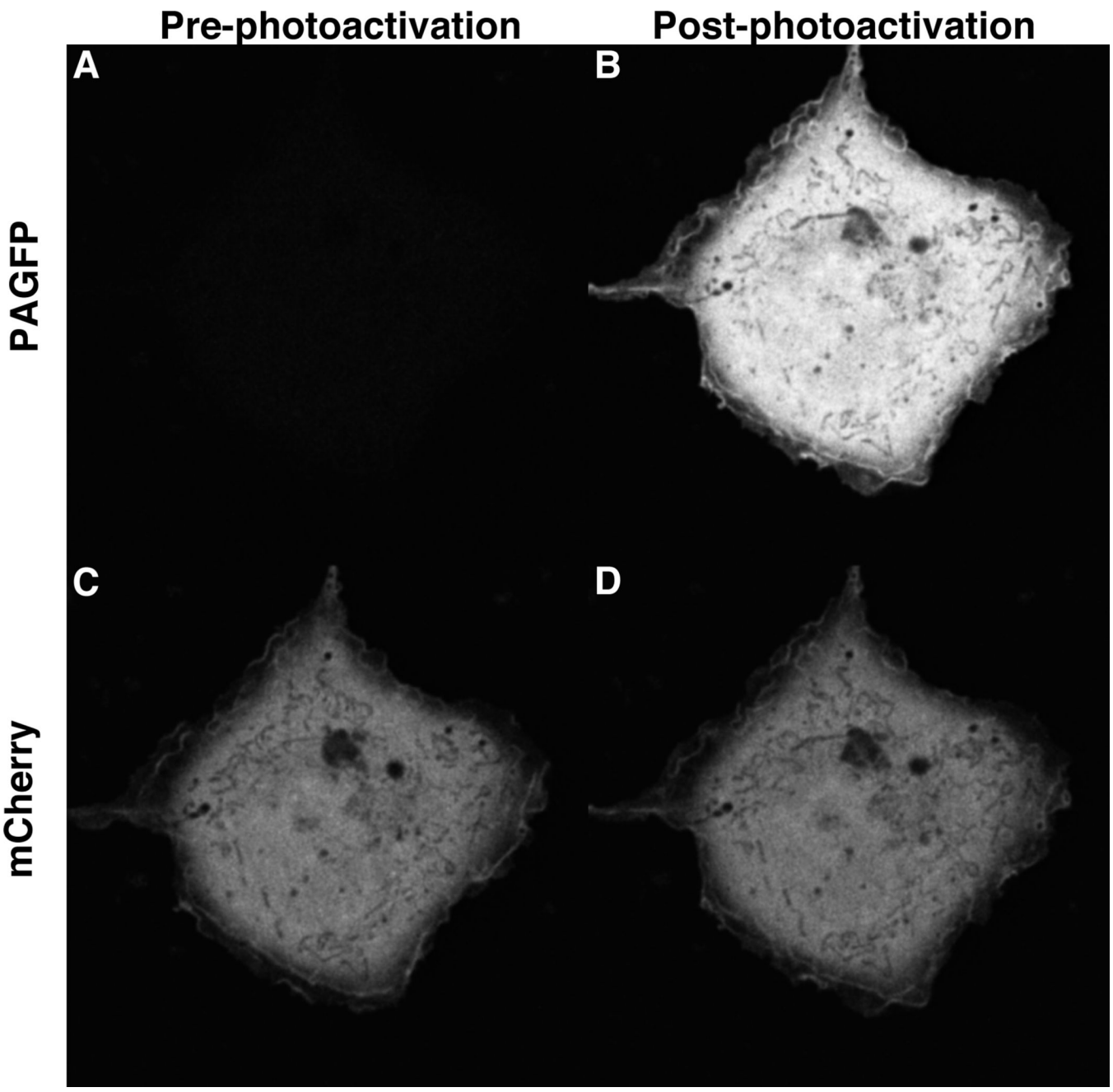

Figure 2.

Table 1

Selected fluorescent protein optical highlighters

Protein	Wavelengths (nm)[a] λ_{em}	λ_{em}	Class	Fold contrast (post/pre)[b]	Extinction coefficient (M^{-1} cm^{-1})	Quantum yield	Reference
PA-GFP	400 (Pre) 504 (Post)	515 517	Photoactivatable	70	20,700 17,400	0.13 0.79	(Patterson & Lippincott-Schwartz, 2002)
PS-CFP	402 (Pre) 490 (Post)	468 511	Photoactivatable	300 green increase 1,500 green to cyan ratio	34,000 27,000	0.16 0.19	(Chudakov et al., 2004)
PS-CFP2	400 (Pre) 490 (Post)	470 511	Photoactivatable		43,000 47,000	0.20 0.23	
PAmRFP1-1	578 (Post)	605	Photoactivatable	70	10,000	0.08	(Verkhusha & Sorkin, 2005)
PAmCherry1	564 (Post)	595	Photoactivatable	4,000	18,000	0.46	(Subach et al., 2009)
PATagRFP	562 (Post)	595	Photoactivatable		66,000	0.38	(Subach et al., 2010)
Kaede	508 (Pre) 572 (Post)	518 582	Photoconvertible	2,000 red to green ratio	98,800 60,400	0.80 0.33	(Ando et al., 2002)
Kikume Green-Red (KikGR)	507 (Pre) 583 (Post)	517 593	Photoconvertible	2,000 red to green ratio	28,200 32,600	0.70 0.65	(Tsutsui et al., 2005)
mKikGR	505 (Pre) 580 (Post)	515 591	Photoconvertible		49,000 28,000	0.69 0.63	(Habuchi et al., 2008)
EosFP	506 (Pre) 571 (Post)	516 581	Photoconvertible		72,000 41,000	0.70 0.55	(Wiedenmann et al., 2004)
mEosFP	505 (Pre) 569 (Post)	516 581	Photoconvertible		67,200 37,000	0.64 0.62	(Wiedenmann et al., 2004)
tdEosFP	506 (Pre) 571 (Post)	516 581	Photoconvertible	200	84,000 (×2) 33,000 (×2)	0.66 0.6	(Wiedenmann et al., 2004)
mEos2	506 (Pre) 573 (Post)	519 584	Photoconvertible		56,000 46,000	0.84 0.66	(McKinney et al., 2009)
Dendra	486 (Pre) 558 (Post)	505 575	Photoconvertible	4,500 red to green ratio	21,000 20,000	0.72 0.70	(Gurskaya et al., 2006)
Dendra2	490 (Pre) 553 (Post)	507 573	Photoconvertible	300	45,000 35,000	0.50 0.55	
Dronpa	503 (ON)	518	Photoswitchable		95,000	0.85	(Ando et al., 2004)
Dronpa-2	486 (ON)	487	Photoswitchable		56,000	0.28	(Ando et al., 2007)

Protein	Wavelengths (nm)[a] λ_{em}	λ_{em}	Class	Fold contrast (post/pre)[b]	Extinction coefficient (M^{-1} cm^{-1})	Quantum yield	Reference
Dronpa-3	487 (ON)	514	Photoswitchable		58,000	0.33	(Ando et al., 2007)
rsFastlime	496 (ON)	518	Photoswitchable	67	39,100	0.77	(Stiel et al., 2007)
Padron	503 (ON)	522	Photoswitchable	143	43,000	0.64	(Andresen et al., 2008)
bsDronpa	460 (ON)	504	Photoswitchable	17	45,000	0.50	(Andresen et al., 2008)
rsCherry	572 (ON)	610	Photoswitchable	6.7	80,000	0.02	(Stiel et al., 2008)
rsCherryRev	572 (ON)	608	Photoswitchable	20	84,000	0.005	(Stiel et al., 2008)
IrisFP	488 (Pre) 551 (Post)	516 580	Photoconvertible / Photoswitchable		52,200 35,400	0.43 0.47	(Adam et al., 2008)

[a] (Pre) and (Post) indicate before and after irradiation to turn on fluorescence, respectively. (ON) indicates the fluorescent state of the protein.

[b] Reported here if indicated in the original reference.