Phototransformable fluorescent proteins: Future challenges.

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Phototransformable fluorescent proteins: future challenges

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Abbreviations:

dSTORM, Direct stochastic optical reconstruction microscopy; (E)GFP, (Enhanced) green fluorescent protein; FP, Fluorescent protein; LOV, Light, oxygen, or voltage-sensing flavoprotein; MEA, Monoethanolamine; PAFP, Photoactivatable fluorescent protein; PA-GFP, Photoactivatable green fluorescent protein; PALM, Photoactivated Localization microscopy; PCFP, Photoconvertible fluorescent protein; Pfr, Phytochrome far-red; Pr, Phytochrome red; Pol II, RNA polymerase II; PTFP, Phototransformable fluorescent protein; RESOLFT, Reversible saturable optical fluorescence transitions; RFP, Red fluorescent protein; RNA, Ribonucleic acid; RSFP, Reversible switchable fluorescent protein; CTPALM, Time-correlated photoactivated localization microscopy; CTPA, Consecutive Two-photon absorption; UV, Ultra-violet; XFEL, X-ray free electron laser

Summary

In fluorescence microscopy, the photophysical properties of the fluorescent markers play a fundamental role. The beauty of phototransformable fluorescent proteins (PTFPs) is that some of these properties can be precisely controlled by light. A wide range of PTFPs have been developed in recent years, including photoactivatable, photoconvertible and photoswitchable fluorescent proteins. These smart labels triggered a plethora of advanced fluorescence methods to scrutinize biological cells or organisms dynamically, quantitatively and with unprecedented resolution. Despite continuous improvements, PTFPs still suffer from limitations, and mechanistic questions remain as to how these proteins precisely work.

Introduction

Fluorescent proteins are central to biology, allowing specific, multicolor and nondisruptive labeling in living cells, tissues and whole organisms [1,2]. Yet, they display highly complex photophysics. For example, it was shown that GFP itself can undergo photoactivation [3], photoconversion [4] and photoswitching [5], in addition to blinking [6], and photobleaching [7]. These low-yield processes have mostly been perceived as nuisances, until the discovery of fluorescent proteins from Anthozoa species completely changed our view. Starting with Kaede in *Trachyphyllia geoffroyi* and EosFP in *Lobophyllia hemprichii*, well-behaved green-to-red photoconvertible FPs soon suggested a huge potential for dynamical tracking experiments and super-resolution localization microscopy, and were rapidly developed into a whole palette of highly performing variants. Likewise, after the discovery of the reversibly switchable asFP595 in *Anemonia sulcata*, the engineering of monomeric Dronpa from *Echinophyllia sp.* revolutionized the field, and was followed by the release of many variants opening up a continuously growing panel of applications, from biotechnology to optogenetics. In the meantime, GFP itself was turned into a highly efficient photoactivatable protein named PA-GFP and different hues followed, adequate for e.g. pulse-chase microscopy approaches. PTFPs developed in the last two years are summarized in **Table 1**. The present paper mainly focuses on future challenges in the field of PTFPs. For a comprehensive view on PTFP's mechanisms, engineering and applications, the reader is directed to the many exhaustive reviews published recently [1,2,8-12]. We start by

recapitulating the three different classes of PTFPs, emphasizing recent mechanistic insight. Then, based on three fascinating examples from recent literature, we highlight important flaws that PTFPs still face today. We list a series of mechanistic questions still to be investigated, and we propose perspectives for further developments of PTFPs.

PTFPs classes and mechanisms

In PTFPs, three main classes are distinguished. Photoactivatable fluorescent proteins (PAFPs) undergo non-reversible light-induced activation from a non-fluorescent to a fluorescent state; photoconvertible FPs (PCFPs) exhibit non-reversible conversion between two fluorescent states with distinct emission colors; and reversibly switchable FPs (RSFPs) can be photoswitched back and forth between a fluorescent on-state and a non-fluorescent off-state. In PAFPs and PCFPs, the phototransformations involve covalent modifications of the FP whereas in RSFPs only conformational rearrangements of the chromophore and its environment take place. The basic mechanisms involved are illustrated in **figure 1**. It should be noted that PCFPs and RSFPs of anthozoan origin often share remarkably high sequence identity and structural similarity. This allowed the development of PTFPs combining both photoconversion and photoswitching properties [13] and the re-engineering of PCFPs into RSFPs [14] or vice versa [15].

PAFPs: photoactivatable fluorescent proteins

PATagRFP [16] and PAmKate [17] are the latest introduced PAFPs, with a red (595 nm) and far red (628 nm) fluorescence emission in their activated state, respectively. Despite a relatively low brightness, these two proteins could successfully be used in multicolor localization microscopy experiments [16,17]. Whereas the photoactivation mechanisms of PA-GFP and PAmCherry are relatively well-established, involving decarboxylation of the fully conserved Glu222 through photo-Kolbe reactions, those of PATagRFP and PAmKate remain to be deciphered. In PATagRFP, the presence of oxygen is required to promote chromophore oxidation, like in PAmCherry. The reaction involves uncharacterized intermediate states and the consecutive absorption of twonear-UV photons (CTPA, e.g. 405 nm) [16], not to be confused with the simultaneous absorption of two photons, commonly referred as TPA. In

fact, Glu222 decarboxylation in PA-GFP is also mainly a CTPA process [18,19], raising the question how weak 405-nm light still activates PA-GFP. Interestingly, Glu222 decarboxylation can cause photobleaching in some anthozoan PCFPs or RSFPs [20], instead of photoactivation, which suggests the existence of two parallel photoactivation mechanisms.

PCFPs: photoconvertible fluorescent proteins

Whereas well-known green-to-red PCFPs emit below 600 nm in their photoconverted state, the recently introduced PSmOrange [21] and PSmOrange2 [22] shift their emissions from orange to the dark red region (660 nm) upon illumination at 488 nm. The photoconversion mechanisms of these two proteins remain to be precisely established but appear to involve CTPA, like PATagRFP, as well as the presence of oxidizing agents in the nanoenvironment. This is in stark contrast to the mechanism of Kaede- or Eos-like PCFPs, in which backbone breakage and elongation of the chromophoric π-system results from absorption of a single UV-violet photon and can be prevented by addition of electron acceptors in the medium [23]. The engineering of a least-evolved ancestral PCFP [24] and ultrafast spectroscopic investigations [25,26] contributed to clarify the mechanism by which green-to-red PCFPs photoconvert. Evidence is growing that a histidine-containing and protonated chromophore embedded in a "typical" anthozoan-FP chromophore pocket does not guarantee efficient photoconversion and that the local charge network [24] as well as global conformational dynamics e.g. due to the oligomeric structure of PCFPs [15] play key roles. In Dendra2, the mechanism by which the anionic chromophore may also promote photoconversion remains unclear. Of major concern is the observation that photoconversion in green-to-red PCFPs is always incomplete (meaning that only a fraction of PCFPs in a sample is able to achieve photoconversion) [27], complicating quantitative molecular counting using PALM (see [9] and **Figure 2b**). The mechanistic reason, and a possible cure, for such incomplete photoconversion are still lacking.

RSFPs: reversibly switchable (or photochromic) fluorescent proteins

RSFPs offer the widest range of applications [10,12], and therefore many efforts have been devoted recently to extend their panel [13-15,22,28-31] and tackle their mechanisms

[15,20,32-35]. Photoswitching in RSFPs is generally based on an intrinsic property of theisolated chromophore: light-induced *cis-trans* isomerization. Therefore photoswitching tends to naturally occur to some extent in many FPs, and has been successfully expanded by engineering in both hydrozoan and anthozoan RSFPs. Depending on whether photon absorption at the peak excitation wavelength induces off-switching, on-switching, or has no switching effect, RSFPs are referred to as negative [36], positive [37], or decoupled [38], respectively, each having their pros and cons. Recent findings concern the heavily debated mechanism by which chromophore isomerization couples to chromophore protonation in negative or positive anthozoan switchers. Several pieces of evidence from ultrafast IR and UV-vis spectroscopy now suggest that (de)protonation of the chromophore in Dronpa [35] or Padron [33] is a ground state process that follows isomerization, although other authors disagree [34]. Another interesting finding is that negative switchers such as Padron are capable of efficient photoswitching at cryogenic temperatures [32], potentially opening up nanoscopy applications at such low temperatures. Recent engineering efforts have also aimed at increasing the switching speed and fatigue resistance (i.e. the number of achievable photo cycles before photobleaching) of RSFPs of use for e.g. RESOLFT applications [28,39] (see below and **Figure 2c**). Blue and infrared RSFPs have been developed that, despite their low brightness, offer exciting perspectives for extracting weak signals by demodulation [29] or difference imaging (see [40] and **Figure 2a**) in conditions of high autofluorescence.

PTFPs applications

PTFPs are presently used in a very wide panel of applications [1,2,8-12] that cannot be exhaustively reviewed here. One recent evolution is that attention is now often paid to optimizing PTFPs for several applications instead of only one, allowing to obtain complementary results with a single construct [15,41,42]. In this regard, bi-photochromic PTFPs such as NijiFP or Dendra2-M159A [13] or three-way highlighters like pcDronpa2 [15] are versatile members that may offer novel applications in the future.

In the following, we take advantage of three outstanding papers to highlight three major challenges that PTFPs' research will face in the next years.

Expanding the PTFPs toolbox with infrared-emitting members

Far-red-shifted variants are a necessary ingredient in deep-tissue or live-animal imaging. In a recent tour-de-force, Piatkevitch et al. [40] developed an infrared-RSFP by exploiting the photocycle of phytochromes between red (Pr) and far-red (Pfr) states. The photosensory core domain of a bacterial phytochrome was engineered to provide PAiRFP1 and PAiRFP2, which can be reversibly switched by 660-nm light into a Pfr-like state emitting fluorescence at ~718 nm. Despite their low fluorescence quantum yield and suboptimal contrast ratio (fluorescence in the off state amounts to ~20% of that in the on-state), the two proteins facilitate deep-tissue imaging: the difference between images recorded in the on and off state of the markers can be computed to highlight e.g. a labeled tumor region (**Figure 2a**). In general, far-red and infrared FPs all suffer from a low fluorescence quantum yield (~5% for PAiRFP1/2), possibly due to the many degrees of freedom in elongated chromophores, promoting a multitude of non-radiative de-excitation pathways. Thus far, iPTFPs are therefore inappropriate for single molecule detection and a challenge for the future will be to engineer iPTFPs of high-brightness suitable for localization microscopy.

Mastering Dendra2 photophysics to monitor transient clustering of RNA polymerase in live cells

Localization microscopy has recently turned into a quantitative method to count macromolecules within cells, assess their stoichiometry or monitor their clustering pattern. The work by Cisse et al [43] illustrates this trend, introducing time-correlated PALM (CTPALM) to dynamically measure the clustering of RNA polymerase II (Pol II) in the nuclei of live human cells. By analyzing the temporal sequence of single-molecule detections in individual clusters, it was shown that "nuclear transcription factories" made of Pol II clusters continuously assemble and disassemble (**Figure 2b**). To confidently interpret the results, Cisse et al, however, had to pay critical attention to the complex photophysics of the employed PCFP marker, Dendra2. Like many other PCFPs, Dendra2 stochastically switches to long-lived dark states once photoconverted, which confuses quantitative studies because each molecule may be counted more than once. A number of methods have been proposed to tackle this difficulty, but they are all statistical by nature and thus remain imperfect [9].

There is yet no clear understanding of the exact mechanism giving rise to this slow blinking, and the nano-environmental sensitivity of the process remains to be assessed. A combination of structural [44,45] and single-molecule [46,47] data on mEos2, Dendra2 and IrisFP suggests that a blinking mechanism in anthozoan PTFPs could involve a transient protonation of carbon C_α of the chromophore methylene bridge *via* the nearby Arg66 (**Figure 3a**). Alternatively, the long dark states could relate to a residual propensity to *cis-trans* chromophore isomerization. Another difficulty in Cisse et al is that the exact numbers of Pol II molecules forming the clusters could not be extracted because of the suboptimal photoconversion efficiency of Dendra2: a number of labeled Pol II molecules never get photoconverted and are thus not counted. Elegant methods have been proposed recently to evaluate the problem in various PCFPs [27] and to correct for it [48], but the underlying photophysics remains mysterious.

Thus, challenges for the future will be to better understand the complex blinking and photoconversion behavior of PCFPs and engineer low-blinking and fully photoconvertible variants.

Video-rate super-resolution microscopy with RSFPs

The concomitant development of sophisticated optical setups and dedicated RSFPs has considerably advanced the nanoscopy approach named RESOLFT [28,39,49]. By employing RSFPs with long on- and off-state lifetimes, RESOLFT requires considerably less light than STED nanoscopy to break the diffraction barrier, but at the expense of a reduced time-resolution. However, employing RSFPs with high switching speed such as Dronpa-M159T [39] or rsEGFP2 [28] allowed relatively fast dynamic RESOLFT imaging. Chmyrov et al [49] demonstrated that video-rate RESOLFT nanoscopy could be achieved on very large field-of-views by designing a highly parallelized setup based on orthogonal and incoherently crossed standing light waves. The spectacular images (**Figure 2c**) were obtained with rsEGFP(N205S), a slow switching variant of rsEGFP that, by emitting about twice the number of photons per cycle, allowed obtaining images of excellent signal-to-noise ratio. Slow-switching variants such as rsEGFP(N205S) deliver high quality images but limit the achievable acquisition rate, which in the present case was more than compensated by the parallelized optical setup. Noteworthy, the light intensity and the cellular environment influence the switching

properties of RSFPs [28] and many other photophysical parameters (e.g. brightness, expression level, maturation rate, contrast ratio) determine the achievable image quality. Another critical aspect in RESOLFT is that the resolution enhancement is limited by the fatigue resistance of the RSFP, calling for more photostable RSFPs (and PTFPs in general). Progress in this direction has been achieved by directed evolution [28] and by recent investigations on IrisFP revealing that that two completely different photobleaching mechanisms occur in anthozoan PTFPs [20]. At light levels typical of standard widefield microscopy ($<< 100$ W/cm²), IrisFP photobleaching involves an oxygen-dependent mechanism, whereas at light levels employed in PALM microscopy ($>> 100$ W/cm²) an oxygen-independent CTPA process dominates (**Figure 3b,c**). It remains unknown whether similar scenarios occur in hydrozoan PTFPs such as rsEGFP or at higher light levels typical of RESOLFT nanoscopy.

Overall, challenges for the future will be to rationally design high-contrast-ratio, fast-switching, high-brightness and fatigue-resistant RSFPs.

Further mechanistic questions

Fluorescent molecules are highly sensitive to their environment. While the role of the environment surrounding organic dyes has drawn considerable research efforts, for example to develop dSTORM nanoscopy [50], its importance in PTFPs has been underestimated. Yet, the β-barrel embedding the chromophore in FPs is generally flexible and permeable. Oxygen [16,21,51] and small buffer components such as ethylene glycol [52] can reach the chromophore, while vitamins in the medium may accelerate photobleaching [53]. There is more and more evidence that the efficiency of phototransformations are influenced by the cellular and/or redox microenvironment, as shown for the oxidative redding of EGFP [23,53], the photoconversion of mEos2 [54], or the photoswitching of rsEGFP variants [28]. However, the precise proton and electron pathways, oxygen routes or role of cavities within PTFP structures remain to be determined. Influences of the solvent on the blinking and bleaching behaviors of PTFPs have also been observed, e.g. presence of redox active molecules such as MEA [55]. In all these cases, the barrel could act as relaying networks via H-bonds or redox active amino acids. Further deciphering such mechanisms PTFPs will necessitate the development of new theoretical and experimental approaches. Quantitative *in cellulo* and *in*

vitro investigations of PTFP's photophysics at the single-molecule and ensemble levels should be pursued jointly, possibly in combination with nanofluidic approaches or optimized sol-gel matrices to cleanly control the PTFP nanoenvironment. Modeling methods would have to be adapted to adequately reproduce low yield processes while taking into account environmental factors. Amazing new perspectives for ultrafast structural investigations seem to be ahead by using X-ray free-electron lasers (XFEL) for visible pump/X-ray probe experiments on PTFPs.

Perspectives for future PTFPs

Fluorescence appears much more widespread in oceans than previously thought, as even vertebrates such as fishes have been found to emit fluorescence [56]. Putative FPs of these organisms probably undergo entirely different fluorescence mechanisms than those involved in GFP-like types, thus offering the opportunity for considerable enlargement of the FP toolbox. A recent example is UnaG, a protein of about half the size of GFP, discovered in a freshwater eel and that fluoresces green upon reversible binding of bilirubin [57]. Another alternative to conventional FPs resides in the increasing use of Light, Oxygen, or Voltage-sensing (LOV) flavoprotein domains [58] whose covalently linked flavin chromophore does not require oxygen to maturate. Amongst other advantages, their small size allows the exploration of tight cellular compartments as recently demonstrated for bacterial periplasm [59]. Engineering these new types of FPs so that they become phototransformable represents a future quest.

Yet, existing PTFPs have still much to offer and will continue to be refined by genetic engineering. Underappreciated improvements such as codon optimizations or adaptations of C and N termini are possible solution to rescue non-functional genetic fusions and to explore "FP-hostile" (e.g. acidic or oxidative) organelles or organisms.

PTFPs also need to see their color palette expanded. The incorporation of non-natural amino acids is a promising route as they can form novel chromophores [60,61]. Plotting the brightness of PTFPs versus their emission color (**Figure 4**), it appears that, despite recent efforts [29,40], most PTFPs concentrate nowadays in the green and orange range of emission, leaving the violet/blue and red/far red regions weakly populated. Even more

strikingly, the few PTFPs engineered from yellow FPs such as EYQ1 [62] or Dreiklang [38] ended up with green variants and it appears that, unlike conventional FPs, yellowish PTFPs emitting between 530 and 560 nm are totally missing. This absence is particularly noteworthy since this is precisely the wavelength range where the brightness is expected to be the highest [1] so that the engineering of bright yellow PTFPs remains a challenge.

In conclusion, unfolding the full potential of PTFPs will continue to keep developers busy for a while -provided continued funding is made available- !

Acknowledgments

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Figure captions:

Fig. 1: Basic mechanisms for the three classes of PTFPs. (a) PAFPs: the case of PA-GFP is shown where the Glu222 is decarboxylated and the chromophore is activated to an anionic/fluorescent form (R=serine). (b) PCFPs: the case of EosFP is shown, where photoinduced cleavage of the backbone extends the conjugated system (R=histidine). (c) RSFPs: the case of Dronpa is shown where the chromophore can be photoswitched between a *cis* anionic fluorescent form and a *trans* protonated non-fluorescent form (R=cysteine). Important features are depicted in red.

Fig. 2: Using PTFPs for deep-tissue imaging, counting macromolecules in dense clusters and developing video-rate nanoscopy.

(a) *In vivo* imaging using PAiRFP1. Overlay of bright-field and fluorescence images of a control mouse (left) and mice bearing a 10-day old PAiRFP1-expressing tumor before and after photoactivation. The difference between 'non-photoactivated' and 'photoactivated' images emphasizes the contrast enhancement allowed by PAiRFP1. Reproduced from [40], with permission. (b) PALM image of Dendra2-RPB1 depicting the heterogeneous distribution of Pol II in the nucleus, with zooms on two Pol II clusters. The time-dependent detection of Dendra2 molecules in a Pol II cluster and the corresponding cumulative detection profile are shown. Reproduced from [43] with permission and with data kindly provided by I. Izeddin. (c) Live-cell imaging with parallelized RESOLFT nanoscopy using the N205S-variant of rsEGFP in a living PtK2 cell expressing the keratin19-rsEGFP(N205S) fusion protein. A zoom and a profile showing the high signal-to-noise ratio achieved are shown. Reproduced from [49], with permission and with data kindly provided by A. Chmyrov.

Fig. 3: Phototransformation mechanisms associated with blinking and bleaching in IrisFP. (a) Blinking involves a thermally-reversible photoinduced proton exchange between Arg66 and the methylene bridge of the chromophore. (b) Bleaching under weak-illumination involves an oxygen-dependent mechanism that results in sulfoxidation of the Met159 and trapping of the chromophore in a protonated/non-fluorescent form. (c) Bleaching under strong-illumination conditions results in the decarboxylation of Glu212, conformational

change of the chromophore pocket and disordering of the chromophore in a sp^3 hybridization state (R=histidine). Important features are depicted in red.

Fig. 4: Brightness distribution of conventional FPs and PTFPs as a function of their emission colors. (a) Conventional FPs and (b) PTFPs are represented by points and labels that are colored to the approximate appearance to the naked eye. The dashed bell-shaped curves highlight the general distributions of brightness. This figure is an extended version of a figure inspired by [1]. Note that a few labels are omitted for sake of readability and that PTFPs are represented in their activated form only. The absence of PTFPs emitting in the 530-560-nm window is apparent. The histograms (insets) represent the distribution of FPs and PTFPs as a function of their emission wavelengths (bin-width = 15 nm).

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Table 1 – Properties of important PTFPs developed during the last two years.

Protein (oligomerization)	Parent protein [wild-type]	Source organism	Chromo-phore	λ_{max} ex/em (nm)[a]		ε (mM^{-1}.cm^{-1})[a]		Φ_{fluo} [a]		Brightness [b]		pK$_a$		Actinic light (nm)[c]		Ref.
PHOTOCONVERTIBLE FLUORESCENT PROTEINS (PCFPs)				*PRE*	*POST*	*PRE*	*POST*	*PRE*	*POST*	*PRE*	*POST*	*PRE*	*POST*			
mEos3.1 (M)	mEos2 [EosFP]	*L. hemprichii*	HYG	505/513	570/580	88.4	33.5	0.83	0.62	218%	62%	5.2	6.0	405		[31]
mEos3.2 (M)	mEos2 [EosFP]	*L.hemprichii*	HYG	507/516	572/580	63.4	32.2	0.84	0.55	159%	53%	5.4	5.8	405		[31]
mMaple (M)	mClavGR2	*Clavularia sp.*	HYG	489/505	566/583	15	30	0.74	0.56	33%	50%	8.2	7.3	405		[41]
pcDronpa (T)	Dronpa [22G]	*Echinophyllia sp. SC22*	HYG	505/517	569/581	115	75	0.85	0.68	291%	190%	5.5	6.3	405		[15]
pcDronpa2 (T)	Dronpa [22G]	*Echinophyllia sp. SC22*	HYG	504/515	569/583	100	105	0.83	0.68	248%	213%	5.8	6.1	405		[15]
PSmOrange2 (M)	mOrange [DsRed]	*Discosoma sp.*	TYG	546/561	619/651	51	18.9	0.61	0.38	95%	23%	6.6	5.4	488		[22]
REVERSIBLY SWITCHABLE FLUORESCENT PROTEINS (RSFPs)				*PRE*	*POST*	*PRE*	*POST*	*PRE*	*POST*	*PRE*	*POST*	*PRE*	*POST*	**on-off**	**off-on**	
(-) modBFP (M)	mKalama1 [GFP]	*A. victoria*	SYG	390/455	NA	27	NA	0.33	NA	26%	NA	5.5	ND	405	514	[29]
(-) mIrisGFP (M)	IrisFP [EosFP]	*L. hemprichii*	HYG	488/516	NA	47	18	0.63	NA	89%	NA	5.3	>10	473	405	[34]
(-) mGeos-M (M)	mEos2 [EosFP]	*L. hemprichii*	MYG	503/514	NA	51.6	NA	0.85	NA	131%	NA	4.5-5.0	ND	488	405	[14]
(-) rsEGFP2 (M)	EGFP [GFP]	*A. victoria*	AYG	478/503	NA	61	NA	0.30	NA	~60%	NA	ND	ND	488	405	[28]
(-) rsCherryRev 1.4 (M)	rsCherryRev [DsRed]	*Discosoma sp.*	MYG	572/609	NA	ND	NA	ND	NA	ND	NA	ND	5.5	592	430	[30]
(+) PAiRFP1 (D)	AtPCD [AtBphP2]	*A. tumefaciens C58*	billiverdin	659/703	690/717	48.7	67.1	NA	0.048	9.6%	ND	4.6	5.2	-	660/750	[40]
(+) PAiRFP2 (D)	AtPCD [AtBphP2]	*A. tumefaciens C58*	billiverdin	657/708	692/719	39.5	63.6	NA	0.047	8.9%	ND	4.7	5.4	-	660/750	[40]

Note: The table summarizes the photophysical properties of PTFPs prior (*PRE*) and after (*POST*) their phototransformation. It is noteworthy that in the last two years, to the authors' knowledge, no PAFPs have been developed.

[a]Abbreviations: λ_{max} ex/em, maximum of excitation/emission spectrum; ε, molar extinction coefficient; Φ_{fluo}, fluorescence quantum yield.

[b]Brightness is the product of fluorescence quantum yield and molar extinction coefficient expressed in % of the EGFP brightness.

[c]wavelengths required for irreversible (PCFPs) or reversible (RSFPs) transitions.

M, monomer; D, dimer; T, tetramer; NA, not applicable; ND, not determined. The sign of the photoswitching in RSFPs is depicted by (-) for negative and (+) for positive switching

Figure 1
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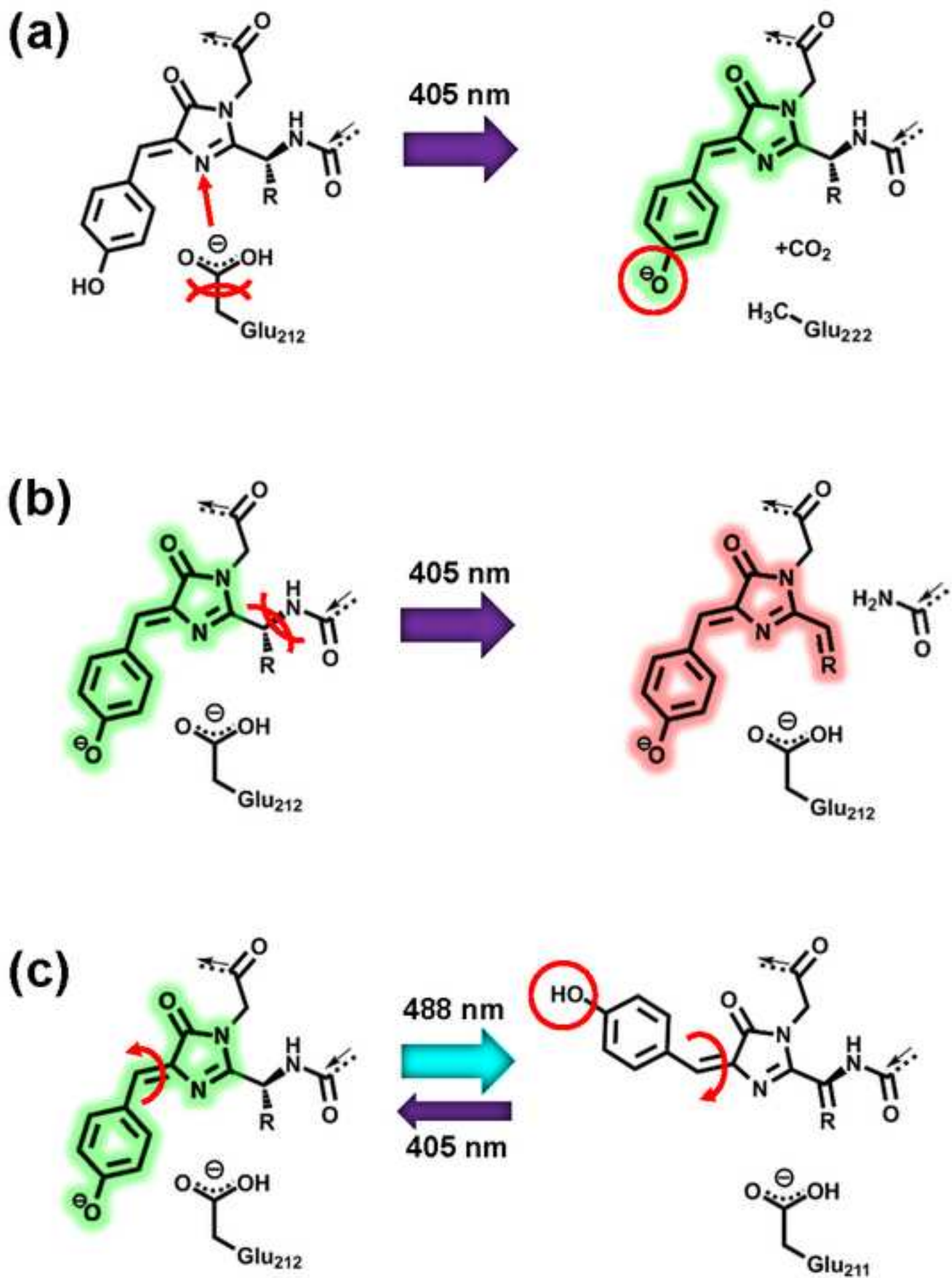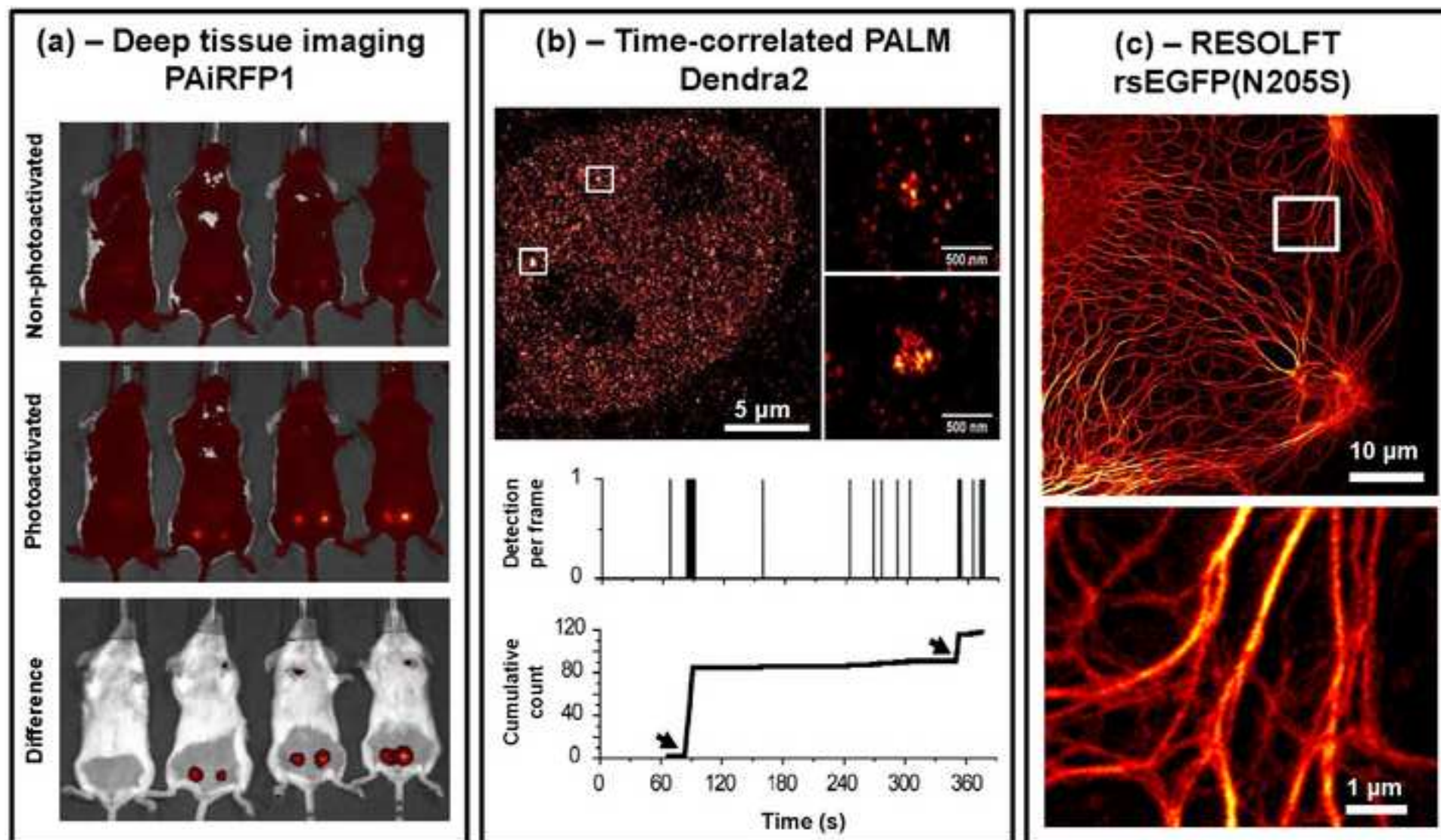**Figure 2**
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(a) – Deep tissue imaging PAiRFP1

(b) – Time-correlated PALM Dendra2

(c) – RESOLFT rsEGFP(N205S)

Figure 3
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Figure 4
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