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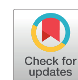
Optimizing the fluorescent protein toolbox and its use



Sam Duwé and Peter Dedecker

Fluorescent proteins (FPs) and fluorescent protein-derived biosensors are indispensable tools in life sciences. They make it possible to probe the location, activity, or interaction of molecules of interest from a subcellular to a multicellular scale. The desire for high-resolution and multidimensional information imposes continuously increasing demands on the performance of the employed probes leading to a continued need for optimization and integration of additional features, such as phototransformable behavior. This review highlights the latest advances in FP engineering, which increase throughput and tailor the improvements directly to the envisioned experiments. Additionally, we discuss recent alternative approaches to introduce or alter phototransformable behavior and describe selected applications of phototransformable behavior in biosensors.

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Introduction

Fluorescent proteins (FPs) are one of the most successful biotechnological toolsets available. These peculiar proteins are indispensable in life sciences and are widely used in imaging experiments ranging from single molecules to entire organisms [1•,2]. Specialized FPs displaying phototransformable behavior — that is irreversible photoactivation (PA)/photoconversion (PC) or reversible photoswitching (RS) (Figure 1) — allow even more complex experiments including photochromic Förster resonance energy transfer (FRET), lock in detection, pulse-chase experiments, and super resolution fluorescence microscopy [3,4]. Furthermore, the fusion of FPs with other proteins and protein domains has led to an impressive amount of genetically encoded biosensors, capable of providing information on protein–protein interactions, the presence or activity of specific molecules and the spatio-temporal regulation of signaling in living systems [5,6]. The biggest advantage offered by FPs and their derived biosensors over organic dye molecules or quantum dots is their inherent genetic encoded nature, which allows for highly specific introduction into the studied system using mostly basic molecular biology and cloning techniques.

Though a significant amount of work has been devoted to creating new and improved FPs, the increasing experimental requirements for example advanced (super resolution) imaging or biosensing experiments create a continued need for improved or specifically tailored FPs. Simultaneously, FP engineering strategies have also evolved in past few years to accommodate this demand. This review discusses a selection of recent exciting developments related to (phototransformable) fluorescent protein optimization and usage.

Choosing and creating the best FPs

The ongoing interest in FP development has resulted in a huge collection of FPs with varying color, brightness, photostability and photochromic behavior. In addition to the spectroscopic and photophysical properties, FP development also influenced the overall biological behavior, including oligomerization tendency, solubility, folding and maturation efficiency, protein turnover, and (mis)localization. However, because there are such a large number of optimizable properties and many of these cannot easily be optimized independently, there is no single ‘perfect’ FP (Figure 2a). The most suitable (combination of) FPs for an experiment will therefore depend on the specific experimental demands and the questions at hand. The sheer number of available FPs can make it difficult to select the appropriate FP or FP combination for a specific experiment. To facilitate this choice, one could look into the available reviews or comparison studies [7–11], or make use of the online database available at www.fpbase.org, which lists the most important characteristics of a large selection of well-known and lesser-known FPs [12].

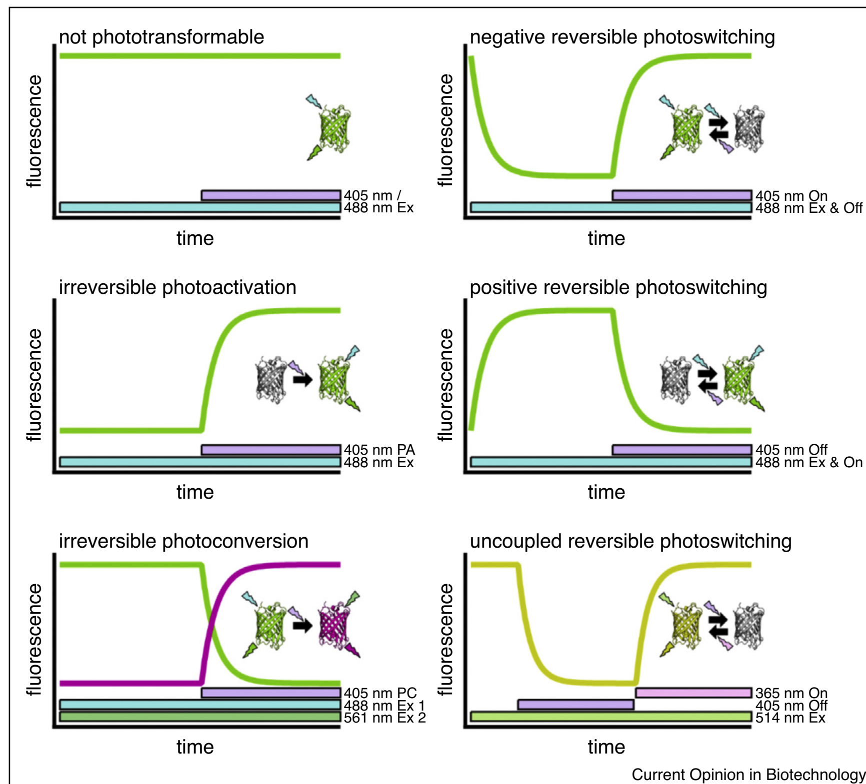
Commonly used FPs have typically undergone one or more optimization attempts. Classically this entailed creating mutants of a promising template, followed by extensive screening of the mutant library expressed in *Escherichia coli*. Interesting mutants were easily selected based on their fluorescence brightness or color, and subsequently characterized in depth (Figure 2b). This strategy has been very widely used in the field. A recent example is the creation of the bright red FP (RFP), mRuby3, a marked improvement over its ancestor

Glossary
AKAP: A kinase anchoring protein
AKAR: A kinase activity reporter
BiFC: bimolecular fluorescence complementation
βME: β-mercaptoethanol
CaMPARI: calcium-modulated photoactivatable ratiometric integrator
Ex: excitation
GFP: green fluorescent protein
GR-GECO: green-to-red photoconvertible genetically encoded calcium indicator for optimal imaging
FC: fluorescence complementation
FLINC: fluorescence fluctuation increase by contact
FP: fluorescent protein
FRET: Förster resonance energy transfer
mt-pcSOFI: multi-tau photochromic super resolution optical fluctuation imaging
PA: photoactivation
PALM: photoactivated localization microscopy
PC: photoconversion
PKA: protein kinase A
refSOFI: reconstituted fluorescence-based super resolution optical fluctuation imaging
RESOLFT: reversible saturable optical linear fluorescence transitions
RFP: red fluorescent protein
RS: reversible photoswitching
SOFI: super resolution optical fluctuation imaging

mRuby2 in terms of brightness and photostability, although it retained the sometimes unwanted photochromic behavior [13•]. The same study also reported the development of mClover3, a highly photostable variant of the bright green FP (GFP) Clover, which is regarded as one of the brightest monomeric GFPs [13•], together with mNeonGreen [14]. Even though this strategy is conceptually straightforward, easy to perform, and has proven its merit in the past, more advanced approaches are required to push FP performance to new heights. Since the low-hanging fruit has likely been picked, manual brightness and/or color screening of randomized mutant libraries may be insufficient to select the next generation of improved FPs. Novel strategies should increase both the throughput and the number of parameters screened in order to retrieve those mutants that display the desired properties.

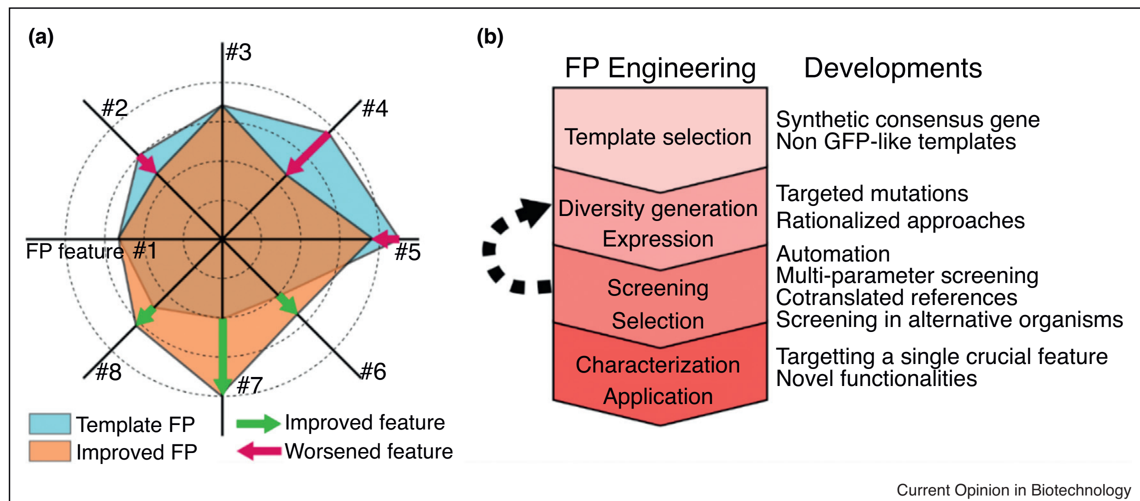
The development of another RFP, mScarlet, already improved upon this classical strategy [15••]. The mutagenesis was performed on a synthetic consensus sequence (mRed7), which incorporated information from multiple RFPs to generate a fitter, monomeric scaffold. Initially,

Figure 1



Conventional and phototransformable fluorescent proteins.
Schematic representation and idealized behavior of the fluorescence of conventional and phototransformable FPs. The fluorescence intensity changes depending on the illumination, indicated by the colored bars. Example wavelengths and the role of the depicted illumination are listed next to the bars. /: no effect, Ex: excitation, PA: photoactivation, PC: photoconversion, On: reversible on-switching, Off: reversible off-switching.

Figure 2



Fluorescent protein optimization.
(a) Optimization of an FP often improves specific features (#6, 7 and 8), while its performance in other aspects decreases (#2, 4 and 5). **(b)** General FP optimization scheme and ways to improve the different aspects discussed in this review. The process often involves repeated rounds of generating diversity and screening/selecting interesting clones, illustrated by the dotted arrow.

the mutants were screened for longer excited state lifetimes, as this is often related to an increased quantum yield of fluorescence and therefore a direct indication of an improved intrinsic brightness. In a next step, mutants were screened for improved brightness and protein maturation using mTurquoise2 as a co-translated reference to compensate for differences in colony size and expression levels. Similar to mRuby3, the resulting mScarlet exhibits higher brightness compared to commonly used RFPs, both intrinsically and in live-cell imaging. Additionally, two mScarlet variants were characterized that trade intrinsic brightness for faster maturation (mScarlet-I) or improved photostability (mScarlet-H). Additional efforts might help to rescue the intrinsic brightness of these RFPs while retaining their remarkable properties.

Other interesting reports include the development of mCarmine, a far-red FP with a 4–5-fold improved brightness over its ancestor mNeptune684 [16•], and the improvement of FusionRed resulting in FusionRed-M, a variant with a twofold increased brightness [17•]. Both optimization attempts introduced elements to automate the screening and selection procedure. The development of mCarmine made use of a screening platform that automated image analysis and colony picking; while the screening of the FusionRed mutants made use of a microfluidic sorter. Especially the second approach opens up a range of new possibilities as the microfluidic sorter is compatible with a variety of prokaryotic and eukaryotic cells and can be used with more complicated screening schemes probing diverse photophysical parameters.

While the optimization efforts discussed above were mostly directed at maximizing the brightness of FPs, several other reports also detail the targeted optimization of other aspects. Gamillus, for example, was developed to have very stable green fluorescence in solutions with pH ranging from 4 to 9 and increased long term stability in acidic or denaturing environments, an area where most green and yellow FPs are lacking [18]. Another example is Citrine2, which was mutated specifically to have twofold increased photostability compared to mCitrine, although it might also exhibit an increased oligomerization tendency [19]. Ultra-stable GFP and monomeric ultra-stable GFP were created with a high resistance to denaturation in sodium dodecyl sulfate at elevated temperatures, particularly for experiments involving optical clearing steps such as volumetric imaging of organs [20]. Several FPs have been optimized recently for use in oxidizing environments, such as the bacterial periplasm [21–23]. As a last example, BrUSLEE was created explicitly with a very short excited state lifetime of approximately 0.8 ns, while preserving 80% of the brightness of EGFP, extending the capabilities of fluorescence lifetime imaging microscopy [24]. Each of these examples tailored the performance of an FP to the emerging experimental needs of advanced and challenging imaging experiments by optimizing one specific property.

New phototransformable FPs

Besides new and improved non-phototransformable FPs, the selection of photochromic FPs has also been expanded recently. The newly developed GMars variants are green RSFPs derived from the green-to-red

photoconvertible mMaple3 [25,26,27•]. Although the GMars variants differ in only one amino acid, they have very distinct properties. One example is GMars-Q, which is particularly well-suited for long-term parallelized reversible saturable optical linear fluorescence transitions (RESOLFT) super resolution microscopy because of its low residual off-state fluorescence and peculiar high resistance to photoswitching fatigue, that is the photobleaching that occurs upon repeated photoswitching [25]. Both beneficial properties can be assigned to efficient shelving of GMars-Q proteins in dark states [28], which unfortunately also introduces additional complexity in the photophysical behavior and limits the amount of detectable fluorescence after repeated photoswitching. GMars-T as a second example was found to have beneficial spectroscopic and photochromic properties for multimodal super resolution imaging, realizing a significant resolution improvement in both super resolution optical fluctuation imaging (SOFI) and RESOLFT experiments [26].

While there are several sets of reliable green RSFPs available, there is a prohibitive scarcity of spectral variation. The usability of orange and red RSFPs in live-cell imaging is mostly limited by their poor switching contrast or unreliable chromophore maturation [29,30]. To address this issue, new red RSFPs have recently been created and shown to be compatible with RESOLFT super resolution imaging. The three reported rsFusionReds exhibited off-switching significantly faster than the previously created mCherryRev1.4 and stable for 20–40 min [31••]. Furthermore, it was shown that the return to the fluorescent on-state did not require damaging UV-violet light but could be efficiently induced by light of 488 nm or even 510 nm, reducing potential phototoxic effects.

Extrinsic factors influence the phototransformable behavior of FPs

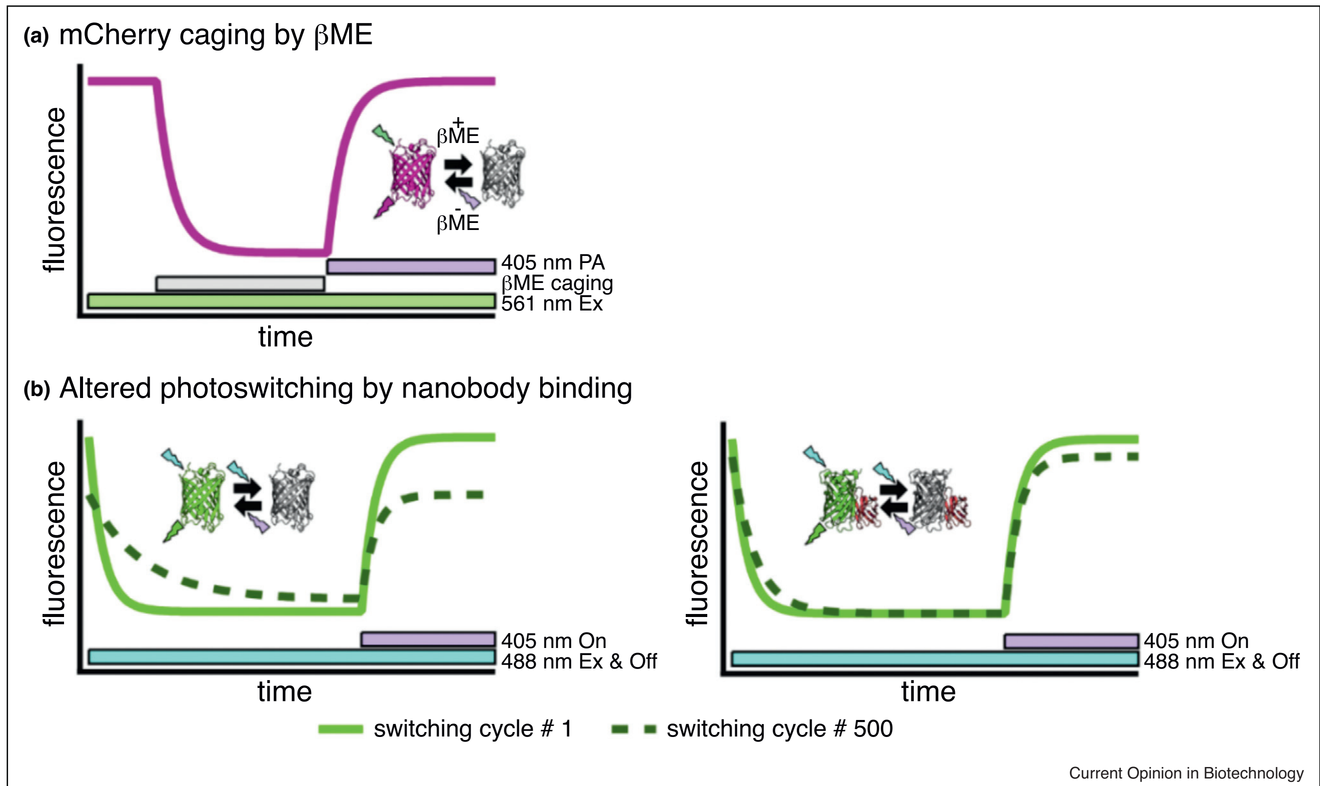
Phototransformable behavior in FPs is mostly dependent on the protein structure, which can be optimized through mutagenesis and screening. However, it is not always necessary to introduce mutations in a FP to induce photochromic behavior, as was shown with the introduction of photoswitching in mCherry and mNeonGreen in the presence of thiols [32]. Additionally, a recent study using mCherry showed that this conventional FP can be chemically caged using β-mercaptoethanol (βME), quenching most of the red fluorescence [33••]. Uncaging of mCherry, and recovery of its fluorescence, could be accomplished through wash-out of the βME or by using 405 nm light as if it were a photoswitchable FP (Figure 3a). A combination of βME washout and 405 nm irradiation induced photoactivation of the caged mCherry molecules useful for single molecule localization microscopy, with photon counts comparable to those of established PCFPs mEos3.2 and mMaple3. Detailed investigation of the underlying mechanism revealed that direct addition of βME to the chromophore and βME induced reduction of the chromophore are responsible for the caging. This direct interaction with the mCherry chromophore is rather surprising, considering that the β-can is believed to shield the chromophore from the environment. Furthermore, the βME caging effect was only seen in mCherry and closely related FPs, indicating it is facilitated by mCherry-specific features. If these features can be identified, through for instance mutagenesis studies, the caging could be enhanced, removed or introduced in other non-phototransformable FPs.

The photoswitching kinetics of RSFPs are also significantly affected by factors other than their amino acid sequence. One such factor is protein flexibility, which is determined by the protein structure but equally by extrinsic elements. The latter is illustrated by the effect of the medium viscosity [34] and the oligomerization state [35] on the RSFP photoswitching rates. Another study illustrated how binding of a specific nanobody (Enhancer [36]) significantly increased off-switching rates, improved resistance to photoswitching fatigue and reduced spontaneous on-state recovery of rsGreen1 and rsGreenF (Figure 3b) [37,38]. Additional investigating suggested that these EGFP derived RSFPs exist in two emissive states, that each harbor their specific photoswitching kinetics. Nanobody binding preferentially stabilizes one of these states and thus alters the overall photochromic characteristics of the rsGreens, which opens up interesting new perspectives. The altered switching kinetics can serve as a contrast mechanism in techniques such as optical lock-in detection [39], τ-RESOLFT [40] or mt-pcSOFI [41], methods that are able to discriminate fluorophores with distinct photochromic behavior. In a similar fashion, the specific change in photoswitching behavior upon binding of a partner molecule can provide an interesting single-color ratiometric readout mechanism for biosensors. These applications would facilitate additional multiplexing by freeing up a large part of the spectrum. Additionally, similar strategies could conceivably alter the phototransformable properties of PAFPs and PCFPs or of spectrally distinct RSFPs, adapting them to the experimental needs. However, this would require the identification of additional FP specific nanobodies or binding peptides.

Phototransformable biosensors

Fluorescent protein-based biosensors are important research tools that report on protein–protein interactions, enzymatic activity, or the presence of specific molecules or ions in living systems. The functionality of these biosensors can be further expanded by the introduction of a phototransformable aspect. This is exemplified by GR-GECO, a photoconvertible Ca^{2+} sensor, which allows to specifically highlight cells of interest during readout of the biosensor [42]. Another example is the integrating genetically encoded Ca^{2+}

Figure 3



Extrinsic factors influence photochromic behavior of FPs.

Schematic representation and idealized photochromic behavior of FPs affected by extrinsic factors.

(a) Photochromic behavior introduced in mCherry through β-mercaptoethanol (βME) caging. Addition of βME quenches the red fluorescence which can be recovered using 405 nm light and/or washout of the βME. PA: photoactivation, Ex: excitation.

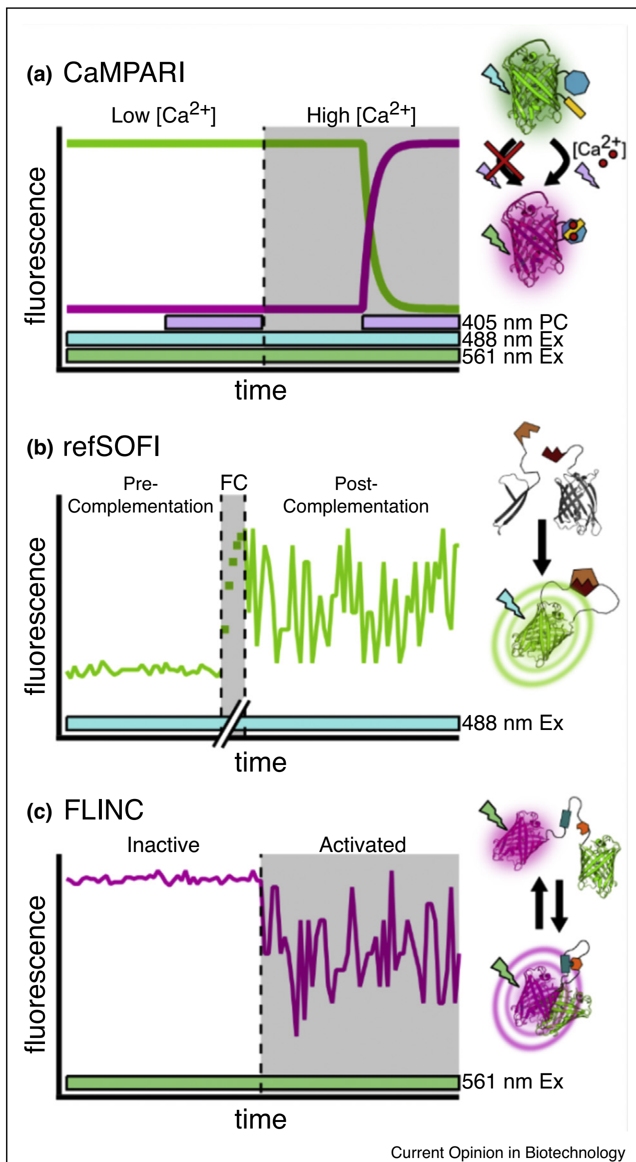
(b) Photoswitching cycle # 1 and # 500 of rsGreen1 (left) and Enhancer nanobody bound rsGreen1 (right). Binding of the nanobody stabilizes the photoswitching kinetics and contrast of the RSFPs. Ex: excitation, On: reversible on-switching, Off: reversible off-switching.

sensor: CaMPARI (calcium-modulated photoactivatable ratiometric integrator) [43]. CaMPARI displays a high photoconversion efficiency in the presence of Ca^{2+} compared to very limited photoconversion in the absence of Ca^{2+}. The selective photoconversion of CaMPARI in cells that experience high Ca^{2+} concentrations during the exposure to photoconverting light has proven to be a valuable tool in the study of active neuronal populations. The performance of CaMPARI was recently improved with the development of CaMPARI2, which exhibits higher contrast and increased brightness [44•].

The introduction of phototransformable aspects in FP-based biosensors also paves the way for super resolution biosensor imaging. This enables resolving the nanoscale compartmentalization of biochemical activity, which is thought to play a crucial role in the regulation of cellular signaling. One strategy to achieve this revolves around bimolecular fluorescence complementation (BiFC) of phototransformable FPs. In essence, two (or more) fragments of the FP are brought together through the interaction of their respective fusion partners and reconstitute to form a functional FP (Figure 4a). When the reconstituted FP exhibits phototransformable behavior, the formation of protein–protein interactions can be visualized with subdiffraction microscopy techniques such as SOFI [45•], RESOLFT [46•], and photoactivated localization microscopy (PALM) [47,48•]. A major limitation of complementation-based approaches is their irreversible nature, precluding the study of protein–protein interaction dynamics [49,50]. Moreover, the creation of suitable complementation fragments of (phototransformable) FPs often involves additional optimization, using mutagenesis and screening, to ensure reliable biological and photophysical behavior upon reconstitution [51].

A recent discovery introduced a new class of biosensors that are able to visualize dynamic activities and interactions in super resolution. The concept hinges on the observation that the conventional RFP, TagRFP-T, displays a large increase in fluorescence fluctuations when it comes into close contact with the green RSFP Dronpa

Figure 4



Phototransformable biosensors.
Schematic representation and idealized behavior of phototransformable biosensors. **(a)** The calcium-modulated photoactivatable ratiometric integrator (CaMPARI) will only photoconvert from the green to the red fluorescent state when exposed to a combination of high Ca^{2+} concentration and 405 nm light. **(b)** In reconstituted fluorescence-based SOFI (refSOFI) two non-fluorescent parts of an RSFP reconstitute a functional fluorophore upon interaction of their respective fusion partners (FC, fluorescence complementation). The RSFP fluorescence fluctuations ('blinking') are used to generate a super resolution image. RSFP complementation is irreversible and not instantaneous. **(c)** In FLINC biosensors a conformational change in the sensing domain brings Dronpa and TagRFP-T into close contact, increasing the fluorescence intensity fluctuations of TagRFP-T useful for generating a super resolution image. The change in TagRFP-T fluorescence is reversible and immediately responds to changes in the sensing domain. PC: photoconversion, Ex: excitation.

[52••] (Figure 4b). Because the FLINC (fluorescence fluctuation increase by contact) effect is dependent on the distance between TagRFP-T and Dronpa, any action that brings both proteins in close contact can be visualized using the super resolution technique SOFI. As a proof of concept, an A kinase activity reporter (AKAR) biosensor was created where the distance between TagRFP-T and Dronpa was regulated by a molecular switch sensitive to protein kinase A (PKA) phosphorylation. Using this approach, PKA signaling microdomains could be visualized in living cells and a role for A kinase anchoring proteins (AKAPs) in structuring these domains was revealed. Because FLINC in TagRFP-T is independent of the Dronpa fluorescence, it is possible to employ a non-fluorescent Dronpa variant, opening up the green portion of the spectral range for multiplexing [52••]. Moreover, it might be feasible to generate small peptides that elicit a similar FLINC effect, which could lead to faster and stronger modulation of the fluorescence fluctuations. Being an entirely new biosensor readout type, additional investigation is needed to probe the potential of FLINC and to increase our understanding of the principles behind the phenomenon. However, innovations like these are crucial in moving biosensor imaging forward into the high spatial and temporal resolution at which cellular processes take place.

Conclusion and outlook

The studies discussed above illustrate the ongoing interest in the optimization and usage of (phototransformable) FPs and biosensors, and simultaneously highlight the diversity present in terms of properties and applications. This variety is actually even larger considering the recent development of extrinsic chromophore-binding RSFPs [53], and the introduction of phototransformable FPs in novel methods such as cryogenic super resolution microscopy [54], optoacoustic imaging [55•] or optogenetics [56••]. The incredibly broad range of applications making use of FPs and biosensors creates an equally broad range of desired properties exhibited by the probes, which establishes a continuous need for optimization. Since the low-hanging fruit has most likely been picked, future FP (biosensor) engineering will require automation using robotics [57] and/or microfluidics [58], and screening in host systems that more closely resemble the final application to increase throughput and match the improvements directly to the envisioned experiments. Alternatively, smart selection of alternative templates and more rational approaches to create specifically tailored probes for different applications can reduce the need to screen huge libraries of mutants [59]. However, to accomplish the latter, a better understanding of the structure–function relationship is required. Time resolved crystal structure determination studies [60,61•] and molecular dynamics simulations [62] could prove very useful in achieving this, as would more comprehensive mutational studies of FPs displaying diverse photophysical and biological behavior [63••]. Taking everything into

consideration, FPs and biosensors will continue playing a crucial role in research. Moreover, it is likely they will continue to surprise us in the future with new functionalities and applications.

Conflict of interest statement

Nothing declared.

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