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Emerging fluorescent protein technologies

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Fluorescent proteins (FPs), such as the *Aequorea* jellyfish green FP (GFP), are firmly established as fundamental tools that enable a wide variety of biological studies. Specifically, FPs can serve as versatile genetically encoded markers for tracking proteins, organelles, or whole cells, and as the basis for construction of biosensors that can be used to visualize a growing array of biochemical events in cells and tissues. In this review we will focus on emerging applications of FPs that represent unprecedented new directions for the field. These emerging applications include new strategies for using FPs in biosensing applications, and innovative ways of using FPs to manipulate protein function or gene expression.

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Introduction

Engineered FPs optimized for live cell imaging have steadily improved over the years, with the introduction of ever-brighter and more red-shifted variants [1–3]. Although advances in alternative fluorophore technologies, specifically organic dyes and quantum dots, has continued apace, FPs will continue to be the fluorophores of choice for most live cell imaging applications for the foreseeable future. The tremendous advantage of FPs relative to these alternative technologies is that they are genetically encoded fluorophores. This unique feature of FPs means that they can be non-invasively introduced into cells in the form of their corresponding gene. In addition, the gene for a FP can be fused with the gene for practically any protein of interest. The chimeric gene can be introduced to live cells, tissues, or transgenic organisms, and the localization and dynamics of the protein of interest visualized by virtue of the inherent fluorescence of the fused FP. The genetically encoded nature of FP is the key feature that allows researchers to precisely monitor activation of gene expression, visualize intracellular protein dynamics, and label subcellular compartments of live cells. Many of these applications are impractical using traditional small molecule tags or quantum dots [4,5].

During this past decade, great strides have been made in transforming FPs from mere genetic intracellular tags into versatile tools with a wide range of applications. We highlight some of the key advances in Table 1. While many researchers continue to work on further improving these established strategies, yet other researchers have been devoting their efforts to restructuring and redesigning FPs to gain novel properties for unprecedented new applications. This review will introduce a number of novel GFP-related technologies that have emerged in recent years. We highlight the innovative engineering and design that gave rise to these new tools, and describe some of the representative applications.

FP-based biosensors incorporating unnatural amino acids

Some of the earliest GFP-based reporters relied on the inherent sensitivity of the chromophore to certain changes in its environment. This sensitivity could be due to either a direct interaction of the analyte of interest with the phenolate moiety of the chromophore (*i.e.* H^+ in the case of a pH reporter), or by binding in a pocket immediately adjacent to the chromophore (*i.e.* Cl^- in the case of a halide reporter) [37–39]. Unfortunately, this approach to biosensor design is relatively limited due to the sparse number of analytes that can interact with, and affect the fluorescence of, the wild-type GFP chromophore.

One approach to expanding the scope of FP-based biosensors is to modify the chromophore such that it gains sensitivity to new analytes of interest. With this goal in mind, Ai and co-workers turned to unnatural amino acid mutagenesis to create single FP-based biosensors that incorporate chemically modified chromophores (Figure 1a). For example, to create a biosensor of hydrogen sulfide gas (H_2S), a gasomediator involved in regulating inflammation, vasorelaxation and cardiac response [40], Ai and co-workers mutated Tyr66 of the chromophore-forming tripeptide to the unnatural amino acid *p*-azidophenylalanine (pAzF) [41•]. Introduction of pAzF, both in *Escherichia coli* and mammalian cells, was performed following the previously reported strategy developed by Schultz and co-workers [42,43]. Specifically, they substituted the codon for Tyr66 of a circular permutated (cp) GFP with the TAG amber stop codon and co-expressed it with a cognate tRNA and tRNA-synthetase.

Table 1

FP-based technology highlights from the past decade

FP-based technologies	FP reporter/FP name	Remarks	References
Cell cycle reporters	FUCCI (Fluorescence Ubiquitination Cell Cycle Indicator)	An FP-based reporter used for imaging cell cycle using chimeras of two different FPs and cell cycle regulators.	[6–8]
Imaging of neuronal structure and connections	Brainbow	A multicolor labeling technology that paints individual neuron in the brain with one of ~100 different hues by random expression of FPs.	[9••,10–12]
Far-red FPs for deep-tissue imaging	TagRFP657	An mKate variant with excitation peak at 611 nm and emission peak at 659 nm.	[13]
	eqFP670	A dimeric far-red FP based from Katushka with excitation peak at 605 nm, emission peak at 670 nm and high photostability.	[14]
	mCardinal	A far-red FP derived from mNeptune. This FP appears particularly promising due to its long Stokes shift, red-shifted emission, and good brightness.	[15•]
Genetically encoded calcium indicators (GECIs)	GCaMP series	Highly optimized Ca^{2+} indicators based on a circularly permuted GFP fused to calmodulin and M13. Additional colors, including red RCaMP, have been reported.	[16–18,25]
	GECO series	A series of GCaMP-type proteins with hues ranging from blue to red. Highlightable, ratiometric, and low-affinity GECOs have also been reported.	[19–24]
Genetically encoded Voltage Indicators (GEVIs)	FRET-based voltage sensors	Voltage indicators based on fusion of the voltage-sensing domain (VSD) from the *Ciona intestinalis* (Ci) voltage sensitive phosphatase to a FP FRET pair.	[26–31]
	Arclight	A voltage indicator based on a fusion of a point mutant of super ecliptic pHluorin to Ci-VSD. Notable for its large fluorescence response to voltage changes.	[32••]
	ASAP1 (Accelerated Sensor of Action Potentials 1)	A voltage indicator composed of a circularly permuted superfolder GFP inserted between S3 and S4 of chicken VSD. Faster on and off kinetics than Arclight.	[33•]
Temporal markers	FP-based timers	Engineered RFPs that change their fluorescence from blue to green to red over time. An alternative design involves a tandem fusion of a fast maturing green protein and a slow maturing red protein.	[34,35]
	TimeSTAMP	Drug controlled reconstitution of a split FP enables visualization of the spatial distribution of a newly synthesized proteins.	[36•]

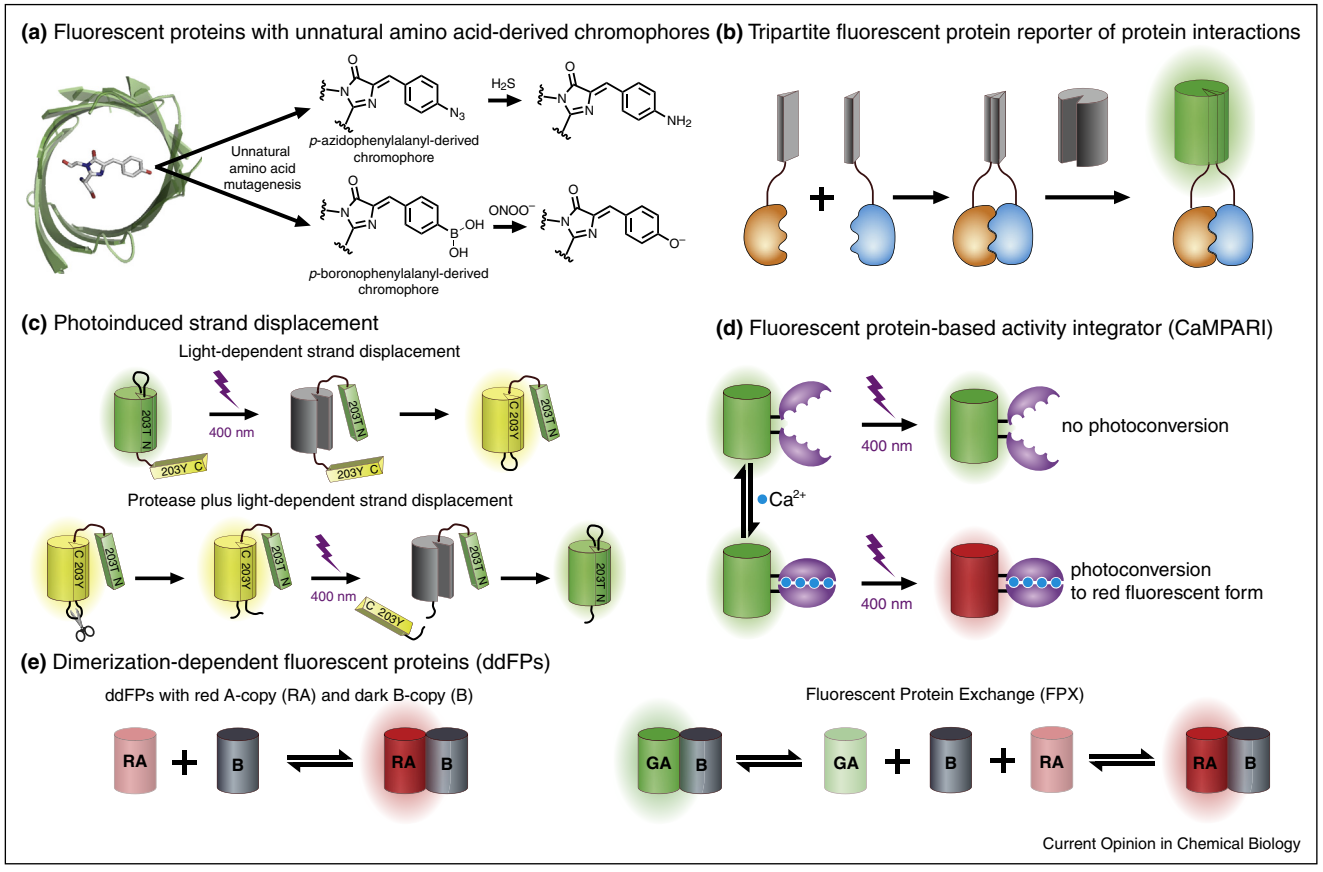
The resulting protein, cpGFP-pAzF, showed only a modest increase in fluorescence when incubated with buffered H_2S both *in vitro* and in live cells. Fortunately, a second-generation biosensor overcame the shortcomings of the first-generation construct, and provides a greater than 10-fold enhancement of fluorescence in response to H_2S [44]. The same group has also reported a FP-based peroxynitrite ($ONOO^-$) biosensor with a chromophore derived from the unnatural amino acid *p*-boronophenylalanine [45].

Potential drawbacks of biosensors that incorporate unnatural amino acids include a slower maturation rate, the possibility of cross-reactivity with non-target analytes, and an irreversible response that hampers their use in monitoring dynamic changes in analyte concentrations. However, this engineering strategy has opened new avenues to broaden the analyte sensitivity of single FP biosensors, especially for biologically important species with no known proteinaceous sensing domains.

Assembling a FP from 3 pieces

Reconstitution of an intact FP from two separate fragments (*i.e.* protein complementation) is an established biosensing strategy has been used to detect protein–protein interactions for well over a decade [46]. Although this strategy has proven effective for detecting and discovering various protein–protein interactions, it can suffer from several shortcomings including poor folding of fragments, undesirable background self-assembly, and the effectively irreversible nature of the complementation [47]. Recently, Cabantous *et al.* [48•] reported a tripartite split GFP-based system that addresses some of the deficiencies of traditional bipartite split FP designs (Figure 1b). This strategy relies on the tripartite reconstitution of GFP β-strands 10 and 11 with the large fragment of GFP composed of β-strands 1–9. Cabantous *et al.* demonstrated the reassembly of functional GFP by fusing β-strands 10 and 11 to an interacting protein pair (FRB/FKBP12 and K1/E1 coiled-coils, respectively) and

Figure 1



Schematic representations of several emerging FP-based biosensor technologies. **(a)** FPs with unnatural amino acid-derived chromophores. **(b)** Tripartite FP reporter of protein interactions. **(c)** Light-dependent strand displacement (*top panel*) and design of a light-dependent protease sensor (*bottom panel*). **(d)** FP-based integrator of Ca^{2+} concentration, which is used as a proxy for neuronal activity. **(e)** Representation of a dimerization-dependent FP (ddFP) (*left panel*) and the ratiometric FP exchange (FPX) strategy (*right panel*).

co-expressing them with the large GFP fragment (β-strands 1–9) in *E. coli*. In comparison to the traditional bipartite complementation, this new system has the potential advantages of higher solubility for fusion proteins and reduced background self-association of the split FP, which thereby decreases the number of false positives.

Light-driven FP-based biosensor

Boxer and co-workers have demonstrated that GFP can undergo photo-induced displacement and exchange of β-strand 10 [49,50] (Figure 1c). This exchange can be used to shift GFP's emission from green to yellow fluorescence due to the presence of Tyr203 (associated with yellow emission) on one copy of β-strand 10 ($s10_{T203Y}$), but not the one that is exchanged with ($s10_{WT}$). To exploit this novel property for biosensing applications, Boxer and co-workers constructed a ratiometric and light-driven protease biosensor [51•]. This was achieved by circularly permuting GFP and flanking the new terminals with either of the two exchangeable β-strands: $s10_{WT}$ or $s10_{T203Y}$. The polypeptide linker connecting the β-barrel and $s10_{T203Y}$ was engineered to include a cleavage site for the protease thrombin (Figure 1c). The relative proportion of the two mature β-barrel isoforms could be tuned to bias one fluorescent hue (green or yellow) by manipulating the length of the two flexible linkers between the barrel and the exchangeable β-strands. Exposure of $s10_{T203Y}$ dominating isoform (90% bound) to thrombin and light *in vitro* gradually shifted its spectral profile from that of YFP to GFP in a span of ~20 min. This biosensor showed a ratiometric fluorescence change of >100-fold, which is much greater than the changes typically observed for FRET-based biosensors.

Fluorescent protein-based activity integrator

The latest generation of GCaMP-type biosensors (Table 1) is highly optimized for *in vivo* visualization of single action potentials in the brains of a range of model organisms [18]. Accordingly, GCaMP and other GECIs are tremendously useful for visualizing the activity of

small regions of the brain over relatively short time periods. However, for many applications it would be desirable to record activity over the whole brain for arbitrarily long periods of time. Unfortunately, there is currently no technology that enables dynamic imaging of neuron activity over the whole brain with sufficient temporal resolution to detect single action potentials, and with single cell spatial resolution.

One feasible solution to the challenge of whole brain activity recording is to develop genetically encoded neuronal activity integrators that provide a fluorescent signal that is proportional to the integrated Ca^{2+} concentration during an experimentally defined window of time. To develop the first example of such a tool, Fosque *et al.* engineered CaMPARI (calcium-modulated photoactivatable ratiometric integrator): a FP that undergoes a Ca^{2+}-dependent green-to-red violet-light dependent photoconversion (Figure 1d) [52]. By expressing this tool in the brains of model organisms, and illuminating with violet light during a defined period of time, neurons that experience higher levels of activity become marked as red fluorescent, while neurons with low levels of activity remain green fluorescent. This marking is effectively permanent and can be imaged *ex vivo* from fixed sections. The authors applied CaMPARI in several model systems, including zebrafish, flies, and mice. One particularly elegant application was the use of CaMPARI for tracing of the olfactory circuitry in adult Drosophila during both optogenetic stimulation and exposure to various odors.

Fluorescent protein exchange (FPX)

Established strategies for converting FPs into active reporters of cellular processes, including protein–protein interactions, kinase activities, and small molecule messenger dynamics, are relatively few in number. For detecting protein–protein interactions, two of the commonly used methods are FRET between two FPs [53,54], and FP complementation [55–57]. Despite their popularity, these strategies suffer from a number of limitations. For example, FRET-based reporters often have relatively small signal changes and correspondingly lower signal-to-noise ratios. Likewise, the slow kinetics and irreversible nature of most FP complementation systems impedes their use for imaging dynamic and reversible protein–protein interactions.

In an effort to address the shortcomings of the current FP-based methods, Alford *et al.* [58•,59] introduced the concept of dimerization-dependent FPs (ddFPs). The development of ddFPs was inspired by the oligomerization-dependent enhancement of red fluorescence of DsRed and other oligomeric Anthozoan-derived FPs. A ddFP pair consists of a quenched fluorogenic FP (A copy) and a non-fluorogenic FP (B copy) that can associate to form a brightly fluorescent heterodimer complex (Figure 1e, *left panel*). The A copy monomer was engineered from dTomato and harbors a fully matured chromophore that is quenched in the monomeric state. The B copy, which was also engineered from dTomato, does not form a chromophore itself but acts as a fluorescent enhancer for A copy upon formation of the AB heterodimer complex. Currently, there are three spectrally distinct pairs of ddFP that have been developed: green, yellow and red. These ddFP pairs have been utilized for intensiometric biosensing of various biochemical activities including Ca^{2+} dynamics, protease activity, and mitochondria–endoplasmic reticulum (ER) membrane proximity. This strategy combines many of the positive attributes of the other methods including the reversible nature of FRET and large fluorescence enhancement of complementation.

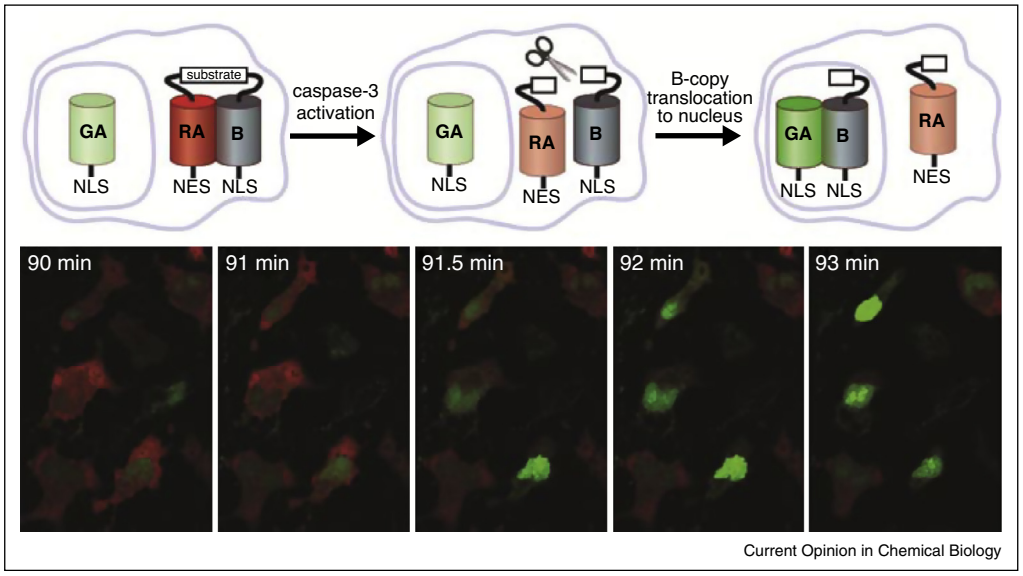
A fortuitous discovery revealed that versions of the B copy optimized to bind to green A copy (GA) and red A copy (RA) partners can pair and induce bright fluorescence with the 'wrong' A partner. That is, a green B copy (GB) can associate with RA and enhance its fluorescence and, similarly, red B copy (RB) can bind and increase the fluorescence of GA. This insight led to the development of the fluorescent protein exchange (FPX) biosensor strategy (Figure 1e, *right panel*). This strategy, as recently described by Ding *et al.* [60••], is based on the swapping of a B copy from RA to GA or *vice versa* in response to changes in cellular events like protein–protein interactions, enzyme activity, and second messenger signaling. As the fluorescence intensity changes from predominately green when GA interacts with B, to primarily red when RA interacts with B, large ratiometric changes in fluorescence can be realized. Accordingly, implementation of FPX for biosensing requires the expression of three ddFP monomers (GA, RA and B) in cells either as separate proteins or as one polypeptide chain.

The FPX strategy has been used for imaging of dynamic changes in Ca^{2+} concentration, cAMP-dependent protein kinase A (PKA) activation, and PIP_2 hydrolysis into diacylglycerol and 1,4,5-triphosphate (IP_3). It was also applied to real-time monitoring of caspase activity during apoptosis with a whole cell change in fluorescence color, either with or without translocation of the B copy from the cytoplasm to the nucleus (Figure 2). These examples establish FPX as a robust and versatile strategy that offers comparable or better qualitative performance than FRET for similar applications. One drawback relative to FRET is that FPX is not readily amenable to quantitative measurements. Compared to use of ddFPs alone, FPX exhibits reduced sensitivity to cell-to-cell variations in the concentration of ddFP monomer units, and is amenable to ratiometric-based biosensing.

FPs as optogenetic actuators

The last decade has seen an explosion of interest in so-called 'optogenetic actuators': molecular tools that can be

Figure 2

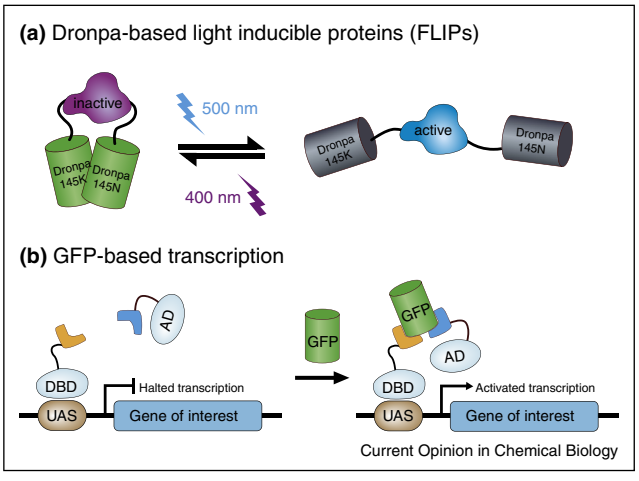


Representative application of the FPX biosensing strategy. *Top panel*: Schematic illustration of detecting caspase activity using FPX plus translocation of the B copy to the nucleus. Upon activation, caspase cuts the protease site on the flexible linker between RA and B harboring a nuclear-localization sequence. The B copy then translocates to the nucleus and binds to GA, forming a green fluorescent heterodimer. *Bottom panel*: Fluorescent images of HeLa cells expressing the caspase biosensor represented above. Cells were treated with staurosporine at $t = 0$ min to stimulate apoptosis (images acquired by Dr. Yidan Ding).

used to artificially manipulate biological activities in time and space through the use of illumination. Such tools can accelerate studies to artificially activate neuronal activity, manipulate protein functions under physiological conditions, and control the expression of specific genes. Important building blocks for the construction of optogenetic actuators are proteins that change their oligomeric state (*e.g.* dimerization of monomers or dissociation of a dimer) upon illumination. Such proteins have been exploited to optically control cellular processes both *ex vivo* and *in vivo* [61].

In 2012, Lin and co-workers [62••] reported that the green fluorescent Dronpa FP variant underwent an illumination-dependent change in oligomerization state. Upon illumination with cyan light (500 nm), the tetrameric Dronpa 145N variant dissociates to form a non-fluorescent monomeric protein. This protein can be converted back to its fluorescent tetrameric state with violet light (400 nm) illumination. This photoswitching process occurs even when two copies of Dronpa are fused as an intramolecular tandem dimer (Figure 3a). This interesting finding led to the development of FP-based optogenetic actuators known as fluorescent light-inducible proteins (FLIPs). To demonstrate the utility of FLIPs for controlling protein activity in mammalian cells, Lin and co-workers caged Cdc42 GEF intersectin and Hepatitis C virus (HCV) NS3-4A protease by fusing interacting Dronpa variants to both termini of the protein. In both cases, the caging strategy led to a decrease in the activity of the target proteins, probably by blocking access to substrates and binding partners. Light-induced uncaging of intersectin led to extension of existing filopodia, while

Figure 3



Schematic representations of FP-based control technologies. **(a)** Light-induced proteins, known as FLIPs, are Dronpa-derived photoswitchable proteins that dissociate from their oligomeric state upon exposure to cyan light (500 nm). Shown here is a caging design using dimeric form of Dronpa. **(b)** The T-DDOG (transcription devices dependent on GFP) strategy uses GFP as a scaffold to control gene expression.

uncaging of HCV protease was detected by release of mCherry linked to the membrane *via* an HCV protease substrate. The FLIP strategy has enabled a new range of applications for FPs and is likely to serve as a versatile complement to established optogenetic dimerizers such as CRY2 and CIB1 [63].

FP-dependent transcription

Tang *et al.* have recently reported a strategy for using GFP to regulate transcription activity [64••]. This strategy was designated Transcription Devices Dependent on GFP (T-DDOG) and relies not on the optical control of GFP, but on the presence of the protein in a given tissue. Essentially, this strategy aims to take advantage of the large number of GFP transgenic animals currently available, and use the presence of GFP to drive other genes of interest. To achieve this aim, Tang *et al.* created a transcriptional system that respond to the presence of GFP by specifically activating certain genes, which could be used to reprogram the development and behavior of transfected cells (Figure 3b). To assemble the T-DDOG system, two optimized anti-GFP nanobodies, derived from the V_HH domains of camelid single-chain antibodies, were separately fused to the Gal4 DNA binding domain (DBD) and p65 activation domain (AD). GFP, serving as a dimerizer, simultaneously binds to both the DBD-nanobody and the AD-nanobody in the nucleus, forming a biologically active assembly that activates gene expression. The nanobody components of T-DDOG are highly specific to *Aequorea* GFP, making this approach suitable for multicolor imaging of additional hues of FPs derived from other species. An element of temporal control was introduced by replacing the DBD with the drug-regulated rTetR DNA binding domain. Application of this strategy *in vivo* directed functional perturbations in specific cell-types in mouse retina and brain.

Although these two emerging strategies, FLIPs and T-DDOG, are still in their infancy, they herald an exciting new era in which the utility of FPs extends beyond its role as a fluorophore. Their development establishes the potential of FPs to be used to manipulate both protein and gene activities in cells.

Conclusion and outlook

A summarized in this review, recent years have seen steady and impressive advances in the development and engineering of FPs and FP-based biosensors. These advances have provided the research community with an ever-improving toolbox of FPs that expands the range of biological questions that can be addressed. Perhaps the most exciting trend in recent years is the growing number of unprecedented, and even surprising, new ways that FPs are being used for research purposes. While it is undoubtedly true that many of these new applications would have been unimaginable just a decade ago, we are certain that the next decade will have no shortage of surprises itself, as protein engineers continue to explore the full potential of FPs.

Conflict of interest statement

Robert E. Campbell and Jhon Ralph Enterina are authors on a patent application describing the fluorescent protein exchange (FPX) strategy.

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- •• of outstanding interest

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