Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Chemical Biology

Emerging fluorescent protein technologies

Jhon Ralph Enterina, Lanshi Wu and Robert E Campbell

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.

Address
Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2, Canada

Corresponding author: Campbell, Robert E (robert.e.campbell@ualberta.ca)

Current Opinion in Chemical Biology 2015, **27**:10–17

This review comes from a themed issue on **Molecular imaging**

Edited by **Samie Jaffrey** and **Atsushi Miyawaki**

http://dx.doi.org/10.1016/j.cbpa.2015.05.001

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