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Review

Optogenetic reporters

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The discovery of naturally evolved fluorescent proteins and their subsequent tuning by protein engineering provided the basis for a large family of genetically encoded biosensors that report a variety of physicochemical processes occurring in living tissue. These optogenetic reporters are powerful tools for live-cell microscopy and quantitative analysis at the subcellular level. In this review, we present an overview of the transduction mechanisms that have been exploited for engineering these genetically encoded reporters. Finally, we discuss current and future efforts towards the combined use of various optogenetic actuators and reporters for simultaneously controlling and imaging the physiology of cells and tissues.

Introduction to optogenetic reporters

Genetically encoded fluorescence

Although fluorescent dyes are essential tools in both classical and cutting-edge structural biology studies, an even more powerful use of fluorescence is provided by fluorescent molecules designed to report biological parameters and their evolution in time. Real-time imaging of biomolecules in live cells using fluorescent probes of structure and function is among the most versatile and widely applied approaches in life sciences. The last 15 years brought a steady stream of technological advances in these techniques, many of which were made possible only by the advent of genetically encoded fluorophores and probe molecules derived from them. These new proteinaceous probes have revolutionised the ability of researchers to monitor protein localisation, small molecule flux, enzymatic activities, the proximity or physical interaction of biomolecules and membrane potential in living tissues.

The genetically encoded fluorophores that sparked this revolution, and that still provide the basis for many of the ongoing developments of optogenetic reporters, are the fluorescent proteins (FPs). FPs are proteins that exhibit visible wavelength fluorescence due to an intrinsic chromophore that forms autonomously from a specific tripeptide sequence within a beta-barrel protein structure (Tsien, 1998). Therefore, the simple introduction of a gene encoding an FP into a live cell bestows that cell with the ability to fluoresce intensely (Inouye and Tsuji, 1994; Chalfie et al., 1994).

Naturally occurring FPs evolved in various marine animal species (*e.g.*, jellyfish and corals) to exhibit properties optimal for the survival of those organisms. However, the biological contexts of most interest to researchers are typically quite different than the natural hosts of these proteins. As a result, naturally occurring FPs often exhibit suboptimal properties under typical experimental conditions and therefore much effort has been dedicated to optimising FPs for use in experimental systems. Fortunately, FPs are particularly amenable to improvement by protein engineering on account of their relatively good expression and accumulation in *Escherichia coli* and because fluorescence phenotypes can be easily screened for in high-throughput formats. Indeed, using standard molecular biology techniques, variants of green fluorescent protein (GFP) with improved folding efficiency were discovered not long after the first demonstrations of its heterologous expression (Crameri et al., 1996; Cormack et al., 1996). Additionally, it was discovered that a small number of mutations could increase the brightness of GFP (Heim et al., 1995). FPs are now routinely engineered to alter their colour, and optimise their photo-physical properties

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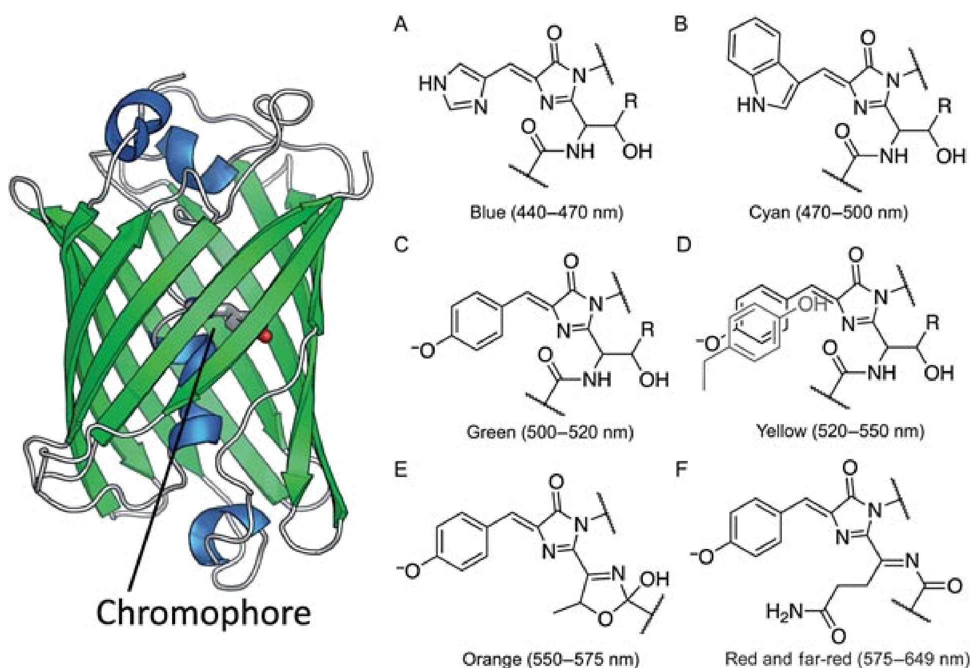
Abbreviations: BiFC, bimolecular fluorescence complementation; BRET, bioluminescence resonance energy transfer; cpFPs, circularly permuted fluorescent proteins; ddFPs, dimerisation dependent fluorescent proteins; FP, fluorescent protein; FRET, Förster (Fluorescence) resonance energy transfer; GFP, green fluorescent protein.

Figure 1 | Representative chromophores for various FP colour classes
Indicated are the general colour classes and their corresponding emission wavelengths.



(such as brightness, photostability and pK_a), as well as to minimise their oligomerisation propensity (Campbell et al., 2002; Shaner et al., 2004; Ai et al., 2006; Shaner et al., 2008; Tomosugi et al., 2009).

Through the combined efforts of researchers who have cloned FP genes from marine organisms, and researchers who have engineered those wild-type FP genes for improved performance in imaging applications, there are so many different FPs available today that it is impractical to list them all and even if one were to do so, the list would quickly become outdated. The best sources of information on available FPs are posted on the internet (*e.g.*, http://www.olympusconfocal.com/applications/fpcolorpalette.html). To aid with classifying this plethora of variants, FPs can be subcategorised into several colour classes based, ostensibly, on the overlap of their fluorescence emission with visible colour bands: blue FPs (BFPs), 440–470 nm; cyan FPs (CFPs), 470–500 nm; GFPs, 500–520 nm; yellow FPs (YFPs), 520–550 nm; orange FPs (OFPs), 550–575 nm; red FPs (RFPs), 575–610 nm and far-red FPs, >610 nm. It should be noted that these colour classes are not in perfect agreement with the normal human perception of colour. For example, the green region of the visible spectrum actually extends to at least 540 nm and orange region to at least 590 nm. This discrepancy is largely for historical regions and is the lasting legacy of the designation of the original red-shifted variants of GFP (with emission at ∼529 nm) as 'yellow' FPs and the coral-derived DsRed (with emission at 583 nm) as 'red'. Figure 1 shows chromophore structures of common FPs from each of the aforementioned classes.

Despite this impressive array of variants, researchers who use FP technology still rely on a relatively small number of preferred FPs for imaging applications. For example, the engineered variant of *Aequorea* jellyfish GFP known as enhanced GFP (EGFP or eGFP) (Yang et al., 1996) and the engineered variant of *Discosoma* coral RFP known as mCherry (Shaner et al., 2004) remain two of the most frequently used variants for one or two colour imaging. Likewise, the vast majority of Förster resonance energy transfer (FRET) experiments with FPs rely on variants of *Aequorea*-derived CFPs and YFPs (Miyawaki et al., 1997).

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A key advantage of the coral-derived FPs is that they extend the spectral range of fluorescence emission covered by *Aequorea*-derived FPs towards longer wavelengths, that is, redder colours. Red colour fluorescence addresses the problems arising from the autofluorescence of biological tissue, which has considerable overlap with the blue/green/yellowish fluorescence of *Aequorea* GFP homologues. Generally speaking, tissue autofluorescence, absorbance (*e.g.*, due to hemoglobin), scattering and phototoxicity, all decrease at longer wavelengths and therefore reddish FPs are preferable to bluish FPs (Deliolanis et al., 2008). This trend holds true through the near-infrared region right up to approximately 1000 nm where the absorbance of water becomes a limiting factor. For these reasons, the wavelength range of approximately 600–1000 nm is often considered the optimal wavelength range for exciting and observing the emission of fluorescent probes (Weissleder, 2001; Ntziachristos, 2006; Deliolanis et al., 2008). Much effort has been invested in engineering ever-longer wavelength FPs and pushing their excitation and emission wavelengths out of the visible range and into the near-infrared (Davidson and Campbell, 2009; Shcherbo et al., 2010; Shcherbakova et al., 2012). Alternatively, researchers can use near-infrared 2-photon (2P) excitation to excite FPs that fluoresce at visible wavelengths (Drobizhev et al., 2011).

Although this review focusses on the use of FPs in more sophisticated reporter constructs, it must be noted that the most common applications of FPs are as passive labels of protein localisation and organelle structure. That is, FPs are most commonly used to tag a protein-of-interest so that it can be tracked spatially and temporally in live cells. This represents a relatively simple strategy and only requires that the FP can be genetically fused to a target protein and expressed in cells with minimal perturbation of the normal structure and function of the fusion partner. Yet another common application of FPs is to use them as reporters of transcriptional activation.

Optogenetic actuators versus optogenetic reporters

Although the definition of 'optogenetics' is arguably somewhat malleable, it seems self-apparent that it refers to techniques in which a combination of light and genetic manipulation is used to perturb, control or analyse the function of cells. These genetic manipulations involve the introduction of genes that encode for chromogenic proteins that are the 'tools' of optogenetics. We further subdivide these tools into the *actuators*: those proteins that have a light-controllable biological function; and the *reporters*: those proteins that provide readouts of biochemical processes occurring in the context of living tissue.

Generally speaking, optogenetic reporters are multi-domain engineered proteins that are designed to respond to a change in their environment with a change in molecular conformation that, in turn, causes a change in fluorescence intensity (intensiometric), fluorescence colour or other spectral properties (often allowing for ratiometric measurements). In other contexts, optogenetic reporters have been called by a variety of other names including genetically encoded sensors, biosensors, nanosensors and indicators. As optogenetic actuators will be reviewed in detail in other articles in this Themed Series, they will not be discussed here.

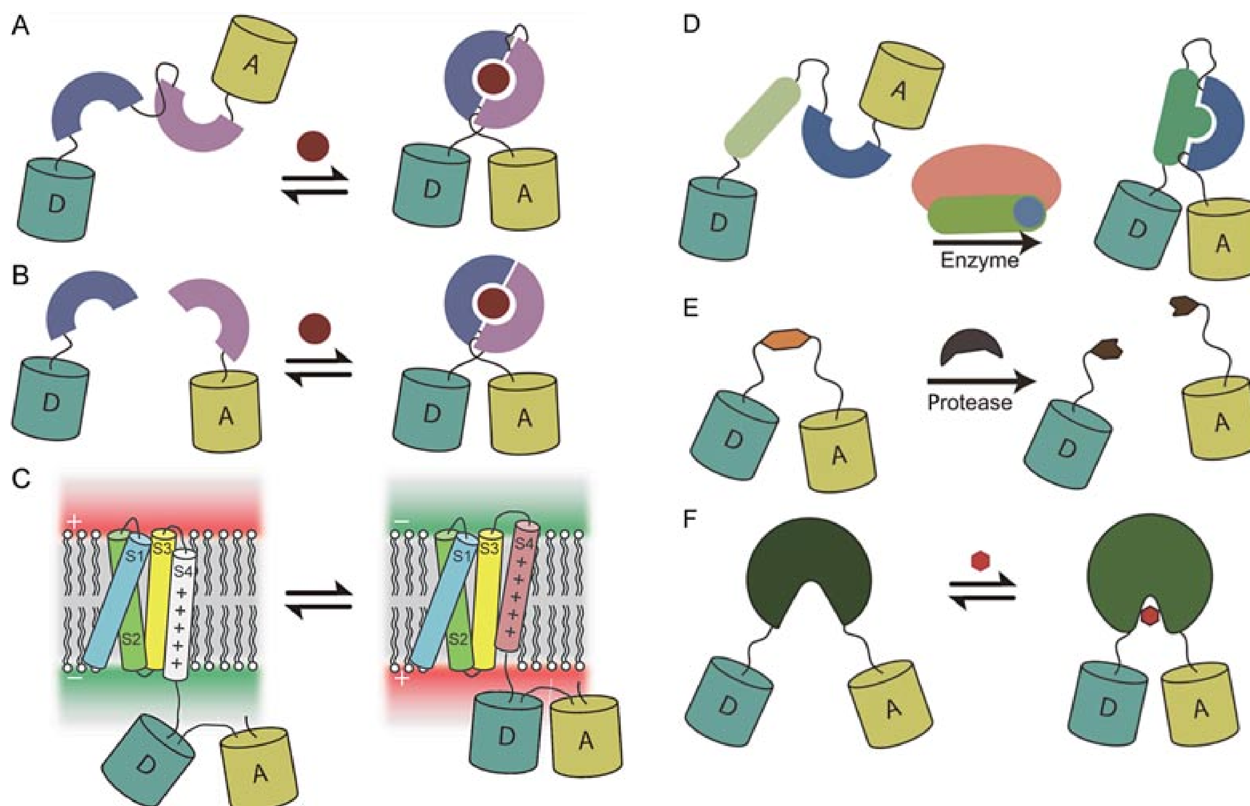
The primary challenge of engineering an optogenetic reporter is transducing a change in molecular conformation of a sensing domain (the part of a protein that is responsive to the biological parameter of interest) into a change in fluorescence properties of the reporter domain (usually consisting of one or two FPs). Fortunately, there are now a handful of well-established transduction mechanisms for converting a wide variety of biochemical changes into either intensiometric or ratiometric changes in FP fluorescence including: FRET (Heim and Tsien, 1996; Miyawaki et al., 1997; Ibraheem and Campbell, 2010), bimolecular fluorescence complementation (BiFC) (Kerppola, 2008) and modulation of FP chromophore environment (single FP reporters) (Siegel and Isacoff, 1997; Baird et al., 1999; Nagai et al., 2001; Nakai et al., 2001). Two recent additions to the set of strategies for designing optogenetic reporters are dimerisation-dependent FPs (ddFPs) (Alford et al., 2012a,b) and the use of engineered microbial rhodopsins (Kralj et al., 2011; Kralj et al., 2012).

Transduction mechanisms of optogenetic reporters

FRET-based reporters

Two FPs with appropriate spectral overlap between the emission of the first FP (donor) and the absorbance of a second FP (acceptor) can engage in a

Figure 2 | FP-based FRET optogenetic reporter design strategies
(**A**) Intramolecular reporters of small molecules. (**B**) Intermolecular reporters of small molecules. (**C**) Reporter of voltage-dependent intramolecular rearrangements. (**D**) Reporters of enzymatic activity. (**E**) Protease reporters. (**F**) Allosteric 'clam-shell' reporters.



distance and orientation-dependent process known as FRET (Campbell, 2009). Practically speaking, the FRET phenomenon causes a quenching of the donor FP fluorescence and an excitation of acceptor fluorescence. The major advantage of the FRET technology is that the optical signal can be split into two detector pathways (corresponding to donor and acceptor fluorescence). The ratio of the fluorescence intensities measured in the two channels ('ratiometric measurement') permits the inherent calibration of quantitative microscopy experiments to minimise cell-to-cell variability in the expression level of optogenetic reporter and intensity changes resulting from out-of-focus movements. The prevailing choice for a FRET donor and acceptor pair is a CFP and a YFP variant, but several other suitable pairs such as mAmetrine–dTomato (Ai et al., 2008) and mOrange2–mCherry (Ouyang et al., 2010), Citrine–mKate2 (Akemann et al., 2010) and Clover–mRuby2 (Lam et al., 2012) have successfully been used in specialised applications. There are several commonly used configurations for the assembly of sensing and FP-based reporting domains in FRET-based reporters. The common theme for all FRET-based optogenetic reporters is that the distance or orientation between a donor and acceptor fluorophore must be modulated in a systematic manner as a function of the biological process of interest. A summary of successful design principles is presented in Figure 2. One strategy to design an optogenetic reporter involves exploiting protein–protein interactions to drive a change in FRET efficiency. These interactions may depend on a small molecule, such as a Ca^{2+} ion, leading to ion indicators implemented as either intramolecular single polypeptide reporters (Figure 2A), or intermolecular reporters (Figure 2B). The archetype of the former is the single polypeptide 'cameleon' reporters for Ca^{2+} (Miyawaki et al., 1997). These reporters are constructed by assembling a four-part fusion protein composed of a FRET donor FP (*e.g.*,

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CFP), the Ca^{2+} binding module calmodulin (CaM), a peptide (termed M13) that forms a complex with Ca^{2+}-loaded CaM, and a FRET acceptor FP (*e.g.*, YFP) (Miyawaki et al., 1997; Persechini et al., 1997). A variation on this design has just the CaM-binding domain of the smooth muscle myosin light chain kinase fused between a FRET pair and can be used to detect Ca^{2+}-loaded CaM (Romoser et al., 1997). The archetypes for the latter intermolecular reporters are the 'split-cameleons' in which one FRET partner is fused to CaM and the other FRET partner is fused to the CaM-binding peptide (Miyawaki et al., 1999). When deployed together, these fusions provide robust sensitivity to Ca^{2+} concentration changes in live cells as the donor and acceptor FPs are brought into close proximity in a Ca^{2+}-dependent manner. The affinity for Ca^{2+} can be adjusted by a variety of approaches including mutation of the CaM domain (Miyawaki et al., 1997), redesigning the CaM–peptide interaction (Palmer et al., 2006) and modification of the CaM to M13 linker length (Horikawa et al., 2010). Another example for FRET-based intermolecular optogenetic reporters of small-molecules is FP-tagged glutamate receptors that report glutamate binding to the dimeric metabotropic glutamate receptor type 1 (Marcaggi et al., 2009).

Instead of driving intramolecular protein–protein interactions by binding of small molecules, certain membrane proteins can undergo voltage-dependent conformational changes, a feature that evolved in the voltage-sensing domains of voltage-gated ion channels (Bezanilla, 2000; Tombola et al., 2006) and voltage-sensitive enzymes (Murata et al., 2005). The voltage-dependent movement of the four transmembrane segments (S1–S4) of these voltage-sensing domains has been exploited in voltage reporters of the FRET-based voltage-sensitive FP variety (Dimitrov et al., 2007; Mutoh et al., 2012; Akemann et al., 2012) (Figure 2C).

Another FRET-based design strategy relies on a post-translational enzymatic modification to induce a conformational change. These optogenetic reporters utilise a substrate domain and molecular recognition domain that binds to the enzymatically modified substrate. The molecular recognition domain and substrate are linked together with donor and acceptor FPs at each end of the polypeptide (Figure 2D). The distance separating the donor and acceptor, as well as the relative orientation of their dipoles, changes in the bound state relative to the unbound state to produce a change in FRET efficiency. The strategy has been used to probe the activity of enzymes, such as kinases (Zhang and Allen, 2007) and GTPases (Aoki and Matsuda, 2009).

To detect the activity of proteolytic enzymes, FRET-based reporters can be designed to have a protease-dependent loss-of-FRET response (Figure 2E). This type of reporter was used in the first demonstration of FRET using FPs in which BFP and GFP were joined by a trypsin-cleavable linker in the context of a single polypeptide (Heim and Tsien, 1996). This particular reporter was limited to *in vitro* cleavage in a test tube. Genetically encoded proteolysis reporters have since been used to detect apoptotic proteases such as caspase-3 (Xu et al., 1998; Ai et al., 2008) or to screen for inhibitors of viral enzymes such as poliovirus 2A protease (Hwang et al., 2008) in live cells.

Yet another FRET reporter design strategy relies on a conformational change of a single protein domain upon binding a small molecule. Periplasmic binding proteins are typically utilised to engineer this class of optogenetic reporter. They are themselves a sensing module that operate on a 'clam-shell' like mechanism whereby two protein domains, linked by a hinge, clamp down on a ligand (Quiocho and Ledvina, 1996; Dwyer and Hellinga, 2004). This changes the proximity and orientation of two genetically linked FPs, thus producing a change in FRET efficiency (Figure 2F). These reporters have been engineered to respond to various analytes such as maltose (Fehr et al., 2002), glucose (Fehr et al., 2003), ribose (Lager et al., 2003), glutamate (Hires et al., 2008) and citrate (Ewald et al., 2011).

The strategies described here represent frequently used design principles for FRET-based optogenetic reporters, but we have not attempted to provide a complete list of all possible variations on this theme and all of the reported implementations. Some particularly interesting, but less frequently used, implementations of FRET-based reporters include ratiometric pH sensitive probes (Esposito et al., 2008) and mechanical tension reporters (Grashoff et al., 2010; Stabley et al., 2012).

Although engineering strategies for FP-based optogenetic reporters typically rely most critically upon the 'sensing' protein modules that are fused to FPs, the FP components themselves can play an active role

in modulation of the FRET efficiency (Nagai et al., 2004; Nguyen and Daugherty, 2005). Of particular relevance in this regard are laboratory-optimised 'sticky' FRET pairs that promote intramolecular complex formation (Nguyen and Daugherty, 2005). This FRET pair can form a weak heterodimer in which the chromophore dipoles are in a near optimal orientation for energy transfer. The sticky FRET partners, known as CyPet and YPet (Nguyen and Daugherty, 2005), were derived from CFP and YFP (both of which are descendants of GFP). It is therefore not surprising that the key mutations of CyPet and YPet that are responsible for their 'stickiness' are located at the GFP homodimer interface (Vinkenborg et al., 2007). This optimised pair tends to exhibit improved sensitivity and higher FRET-efficiency changes relative to the conventional CFP and YFP FRET pair, particularly when used in protease reporters (Figure 2E) (Nguyen and Daugherty, 2005; Vinkenborg et al., 2007).

Single FP-based reporters

Single FP-based optogenetic reporters utilise an FP that is engineered to have a chromophore that is spectrally responsive to its environment or to a protein conformational change that is caused by the analyte of interest. Because only a single FP is involved, this spectral response must necessarily be a non-FRET mechanism, and can be conceptualised as fluorescence quenching or dequenching by any other means. One of the primary advantages of single FP-based reporters relative to FRET-based reporters is that the magnitude of the fluorescence change is typically greater (Baird et al., 1999). Although the majority of single FP-based reporters are intensiometric, ratiometric reporters have also been reported (Zhao et al., 2011). A disadvantage of single FP-based reporters is that the design is not nearly as versatile as the FRET-based strategy, and so a much smaller set of reporter specificities has been successfully engineered to date.

Single FP-based optogenetic reporters can be categorised into two sub-classes. The first class is single copy FPs with inherent biosensing capabilities. These FPs are not fused to any molecular recognition domains or sensing modules, but instead possess an inherent molecular recognition function that cause them to change their output signal in response to a change in their environment. In this case, the beta-barrel structure that surrounds the chromophore usually remains fully intact and so the analyte must either be small enough to penetrate the beta-barrel (*i.e.*, small ions such as H^+ and Cl^-) or otherwise modulate the chromophore environment by exerting influence from the exterior of the beta-barrel (*i.e.*, redox sensitive FPs with exposed disulphide bonds). Typically, the change in the chrompohore environment causes a change in the pK_a of the chromophore by preferentially stabilising either the protonated or deprotonated form. Assuming that the pH remains constant, a change in the chromophore pK_a will cause a change in the proportion of the FP that is in the brightly fluorescent form (typically the deprotonated form), and an intensiometric change in fluorescence will be observed.

Single FP-based reporters have been developed to exhibit sensitivities to pH (Miesenböck et al., 1998), halides (Jayaraman et al., 2000) and redox potential (Ostergaard et al., 2001; Dooley et al., 2004; Hanson et al., 2004). The number of analytes or biological processes that can effectively elicit a change in the optical output of a single FP limits the applicability of this sensing approach. Fortunately, this limitation is overcome in the second class of single FP-based reporters in which the FP is genetically fused to extrinsic biosensing domains, thus extending the range of potential analyte sensitivities.

The second class of single FP-based optogenetic reporters utilises a design in which FPs are fused to an extrinsic molecular recognition module (sensing domain) capable of sensing an analyte or physicochemical parameter of interest. Single FP membrane voltage indicators may be allocated into this class of reporters because their FP likely reports the change of physiochemical environment associated with their voltage-dependent translocation towards the lipid membrane (Perron et al., 2009; Jin et al., 2011). However, in most circumstances conformational changes of a sensing domain has little impact on an attached FP that is protected by the native cage-like beta-barrel structure that surrounds the chromophore. One of the keys to creating single FP-based reporters is engineering the FP such that its chromophore is not fully isolated from the external environment. A well-established method for partially exposing the chromophore is to engineer a circularly permuted FP (cpFPs) in which the original N- and C-termini are joined by a polypeptide linker

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Figure 3 | Single FP-based G-CaMP reporter for Ca^{2+}

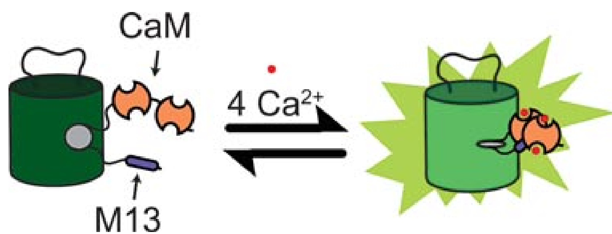
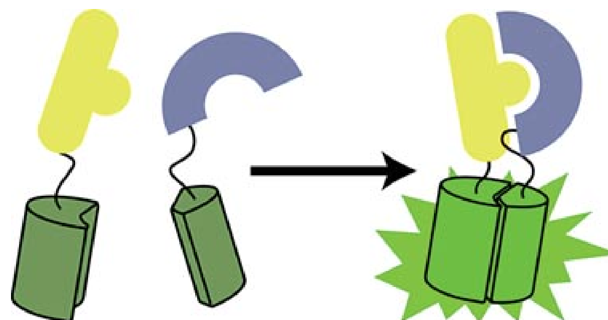


Figure 4 | Bimolecular fluorescence complementation (BiFC) strategy



and new termini are introduced into the side of the beta-barrel in close proximity to the chromophore (Baird et al., 1999; Topell et al., 1999). This effectively opens up a 'hole in the cage' and allows the chromophore to interact with molecular species in the external environment. The effect of the molecular recognition event is to confer, typically through a conformation change, a perturbation to the FP structure or chromophore microenvironment to change its optical output. This mechanism was explored first by Baird et al. (1999) and cpFPs are now most extensively used in G-CaMP-type Ca^{2+} reporters (Nakai et al., 2001; Tian et al., 2009; Zhao et al., 2011) (Figure 3). The molecular recognition elements for these and similar (Nagai et al., 2001) cpFP-based Ca^{2+} sensors are the CaM domain and the M13 peptide that are fused to the N- and C-termini of cpGFP. Crystal structures of G-CaMP in the Ca^{2+}-loaded and Ca^{2+}-free states have revealed the mechanism of the intensiometric response to Ca^{2+} (Wang et al., 2008; Akerboom et al., 2009). In the absence of Ca^{2+}, solvent has free access to the chromophore and quenches fluorescence. When Ca^{2+} is present, the conformational change associated with the binding of Ca^{2+}-loaded CaM to M13 effectively plugs the hole adjacent to the chromophore and restores fluorescence by preventing solvent access (Wang et al., 2008; Akerboom et al., 2009) and fluorescence is restored. For many years these Ca^{2+} reporters were limited to green and yellow fluorescence, but a new series of G-CaMP-type Ca^{2+} reporters, known as the GECO series, was recently engineered to include blue and red variants (Zhao et al., 2011).

BiFC-based reporters

FPs split into two non-fluorescent fragments at an appropriate splitting site can assemble to the native fluorescent beta-barrel structure (Figure 4) (Ghosh et al., 2000; Kerppola, 2008). In the original demonstration of a reporting strategy that exploits this feature, non-fluorescent N- and C-terminal GFP fragments were fused to leucine zipper domains that form functional dimers. The two FP fragments formed a fluorescent complex spectrally indistinguishable from its GFP parent molecule upon dimerisation of leucine zipper domains (Ghosh et al., 2000). As this example demonstrates, the primary use of FP complementation is to visualise protein–protein interactions or compartmentalisation of interactions in live cells (Kerppola, 2008). The strategy requires interacting partners be fused to each of the split FP fragments, as well as achieving sufficient expression levels of the fusions in an aerobic environment. Interaction of the proteins of interest leads to the proximity-induced reconstitution of the inert FP fragments and an intact, and fluorescent, FP is formed. Interactions may be visualised in any cellular compartment provided the fused proteins can gain access to one another. Split FP complementation technology has also been applied in various high-throughput strategies to identify protein interaction partners (Remy and Michnick, 2004; Ding et al., 2006; Kojima et al., 2011). However, significant limitations to this technology include slow kinetics of fluorescence development and the irreversible nature of complementation (Kerppola, 2008).

The BiFC colour palette has now expanded beyond GFP. Several FPs of varying colour hue, from blue to far-red, have now been split and used as complementation-based reporters including: CFP

(Shyu et al., 2006), YFP (Shyu et al., 2006; Ohashi et al., 2012), RFP (Jach et al., 2006; Fan et al., 2008) and a far-red FP (Chu et al., 2009). Furthermore, the availability of multiple colours has enabled the imaging of multiple protein–protein interactions occurring simultaneously in a cell (Hu and Kerppola, 2003; Grinberg et al., 2004).

ddFP-based reporters

In an approach that shares some conceptual similarities with BiFC, Alford et al. (2012a,b) recently reported a new strategy for converting FPs into optogenetic reporters. The key to this strategy is a pair of engineered FPs that exhibits little to no fluorescence in their monomeric state, but exhibit bright fluorescence when allowed to dimerise. This approach is similar to BiFC as a complete functional protein (in this case a functional dimer) is being reconstituted from its parts. However, a key difference between ddFPs and BiFC is that the interaction is reversible in the case of ddFPs but irreversible in the case of BiFC. On the contrary, BiFC provides a much larger change in fluorescence intensity upon interaction. It could be argued that ddFPs also share some similarity with the FRET approach, as two FPs are required and the signal change reflects their molecular proximity. A key contrast with the FRET approach is that the ddFPs retain substantial affinity for each other and will spontaneously dimerise at relatively high intracellular concentrations in the 10's of micromolar. Although some FRET pairs are said to be 'sticky' (*i.e.*, they have a weak tendency to dimerise), the dissociation constants are much higher ($\sim 100\ \mu$M) so spontaneous dimerisation is less of a concern under typical imaging conditions (Zacharias et al., 2002).

Voltage-dependent modulation of microbial rhodopsin fluorescence

Bacteriorhodopsin has been subjected to extensive protein engineering since its discovery in 1971 in *Halobacterium halobium* (Oesterhelt and Stoeckenius, 1971). However, the vast majority of this engineering has focussed on improving and modifying its proton pumping abilities and photo-induced colour changes (Hampp, 2000). It has long been recognised that bacteriorhodopsin exhibits a weak fluorescence that is characterised by a very large Stokes shift (excitation maximum at 560 nm, emission maximum at 750 nm) (Kouyama et al., 1985) and a very low quantum yield of approximately 2×10^{-5} (Alfano et al., 1976). There have, to date, been remarkably few papers that have focussed on this aspect of bacteriorhodopsin, probably because there was no obvious utility or application for this weak fluorescence.

It is only very recently, with the work of Cohen (Kralj et al., 2011), that researchers identified an exciting new application that exploits the weak fluorescence of bacteriorhodopsin: imaging of membrane voltage potential. Specifically, the authors succeeded in imaging the fluorescence of a bacteriorhodopsin homologue (the D97N mutant of proteorhodopsin, designated PROPS) by using a highly sensitive total internal reflectance illumination strategy. Calibration experiments revealed that PROPS undergoes a fivefold increase in fluorescence between membrane voltages of -170 and $+70$ mV. The mechanism of this change is proposed to involve voltage-dependent changes in the pK_a of the ionisable Schiff base that links retinal to a lysine residue in the protein core. Accordingly, PROPS is a promising, but currently limited, approach for imaging of membrane potential in bacteria (Kralj et al., 2011).

Because even extensive engineering efforts have not enabled the expression of PROPS in mammalian cells (Kralj et al., 2011), the Cohen group has also explored the utility of other bacteriorhodopsin homologues. This effort led to the discovery that archaerhodopsin-3 (Arch), a homologue that has similar properties to proteorhodopsin, exhibits good membrane targeting and retains its voltage sensitivity when expressed in mammalian cells (Kralj et al., 2012). As with PROPS, ARCH is limited by an extremely dim fluorescence signal.

The spectral overlap problem

Clearly, the combined use of various optogenetic actuators and reporters is potentially a powerful strategy for simultaneously controlling and imaging the physiology of cells and tissues. However, despite this potential, there remain relatively few examples of such combinations in the research literature. Why has this approach not had a greater impact on research efforts to date? The answer to this question is that spectral overlap between the action spectra of many of the optogenetic actuators (Figure 5) and the excitation spectra of many of the optogenetic reporters (Figure 6) overlap to such an extent that it is

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Figure 5 | Action spectra ranges of representative optogenetic actuators
Ranges indicate the wavelengths at which the action spectrum is greater than 50% of its maximum value. Wavelengths of light outside of these ranges can cause activation, although higher intensities are required as the wavelength distance from the peak value increases.

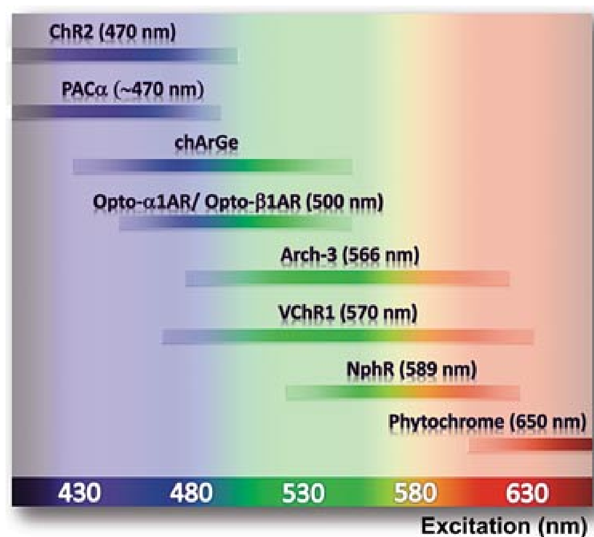
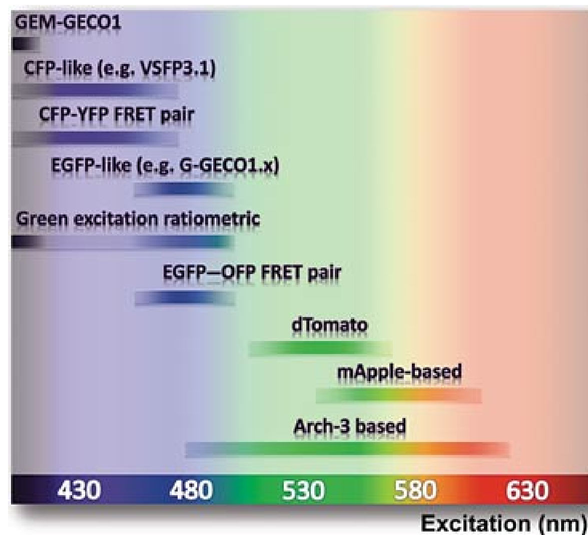


Figure 6 | Excitation ranges of representative optogenetic fluorophores and reporters
Ranges indicate the wavelengths at which the excitation spectrum is greater than 50% of its maximum value. Wavelengths of light outside of these ranges can cause fluorescence, although higher intensities are required as the wavelength distance from the peak value increases.



challenging, if not impossible in many cases, to perform independent imaging of the reporter without causing further activation of the actuator (and vice versa). For example, the action spectrum of ChR2 extends from the ultraviolet through most of the visible region up to approximately 550 nm (Nagel et al., 2003; Zhang et al., 2008; Lin et al., 2009), severely restricting the ability of researchers to image neuronal activation with popular genetically encoded [*e.g.*, cameleons (Miyawaki et al., 1997) and G-CaMP (Nakai et al., 2001)] or synthetic dye-based reporters (Prigge et al., 2010).

Fortunately, the increasingly diverse spectral properties of the optogenetic actuators and reporters already permit some pairings to be used with reasonably low spectral overlap. Generally speaking, the single FP-based reporters are favourable because they occupy a narrower portion of the optical spectrum. For example, mApple-based Ca^{2+} reporters such as the R-GECO1 (Zhao et al., 2011) have a red-shifted excitation and could, in principle, be used with optogenetic actuators such as PAC (Schröder-Lang et al., 2007) and ChR2 (Nagel et al., 2003; Ohkura et al., 2012) that are activated with violet or blue light. Similarly, reporters with blue-shifted excitation such as GEM-GECO1 (Zhao et al., 2011) and FRET pairs with a BFP or CFP donor are good candidates for combined use with actuators that possess red-shifted actuation wavelength like NphR (Zhang et al., 2007), VChR1 (Zhang et al., 2008) and Arch (Kralj et al., 2012).

In addition to spectral overlap, the complex photophysics of reporter FPs must be taken into account when used in conjunction with optogenetic actuators. Many FPs have been reported to exhibit complex photophysical behaviours such as photoactivation, reversible photoswitching and irreversible photoconversion, particularly when illuminated with wavelengths shorter than their primary excitation peak. Such behaviours have now been observed in essentially all colour classes (*i.e.*, cyan, green, yellow, orange and red) of FP (Chudakov et al., 2004; Valentin et al., 2005; Sinnecker et al., 2005; Henderson et al., 2007; Shaner et al., 2008; Kremers et al., 2009). Accordingly, it is expected that FP-based optogenetic reporters will inherit these

complex photophysical traits from their progenitor FPs. Indeed, several groups have now observed some extent of reversible photoswitching behaviour of mApple-derived R-GECO1 under illumination of strong blue light (unpublished observations from the groups of Wen-hong Li, Takeharu Nagai, Thomas Hughes and Robert Campbell). In this particular case, illumination with blue light causes a transient increase in red fluorescence that returns to baseline in a few seconds. Accordingly, researchers should exercise caution when using ChR2 in conjunction with R-GECO1, as increases in red fluorescence could potentially be photophysical artifacts. Control imaging experiments using cells expressing only the reporter are essential. Fortunately, R-GECO1 photoactivation may be minimised by using lower intensity light for ChR2 activation.

The spectral overlap problem also plagues the use of multiple optogenetic reporters to concurrently monitor independent physiological events in the same cell (Carlson et al., 2009). For example, there are only a very few reported examples of spectrally distinct FRET pairs that can be individually excited and imaged for the purpose of multi-parameter imaging. Some successful examples include concurrent monitoring of caspase-3 activity and Ca^{2+} dynamics (Tomosugi et al., 2009; Ding et al., 2011), as well as Src kinase activity in conjunction with matrix metalloproteinase activity (Ouyang et al., 2010). The recent report of the red intensiometric Ca^{2+} indicator R-GECO1 has increased the number of opportunities for using multiple optogenetic reporters, as R-GECO1 and any GFP- or CFP–YFP-based reporter can be imaged with no substantial spectral overlap (Zhao et al., 2011). For example, single cell imaging of diacylglycerol with a GFP-based reporter and Ca^{2+} with R-GECO1 has recently been reported (Tewson et al., 2012).

Prospects for circumventing the spectral overlap problem

Modulating light intensity or illumination frequency

There are, to date, very few examples in which combinations of optogenetic actuators and reporters have been used in a single study. In one such study, Guo et al. (2009) reported a successful mapping of functional neural connections in *Caenorhabditis elegans* by using a combination of ChR2 for neural circuit activation and G-CaMP for Ca^{2+} imaging. Because G-CaMP has an EGFP-like excitation spectra with strongest excitation around 488 nm (Figure 6), and ChR2 has strong absorption in the same region of the visible spectrum with a maximum at 470 nm (Figure 5), any effort to combine these two tools must overcome the spectral overlap problem. The solution identified by the authors was to use a low-power 488 nm laser (0.1 mW/mm^2) to excite G-CaMP and a higher-power 450 nm light source (8 mW/mm^2) to activate ChR2. Apparently, low light levels that are sufficient for extracting sufficient signal from G-CaMP are not sufficient for activating ChR2. By employing this setup, the authors were able to demonstrate a functional connection among ASH, RIM and AVA neurons by photo-activating ChR2 in ASH and RIM neurons and observing increased Ca^{2+} levels in the AVA neuron. The authors speculate that more recent generations of G-CaMP with brighter fluorescence (Tallini et al., 2006; Tian et al., 2009) could produce more robust signals with similar low levels of excitation.

In a second exemplary study, Li and Tsien (2012) used a combination of optogenetic actuators and reporters to achieve optical control of neurons and monitor their activity. To study synapse-specific regulation in cultured hippocampal neurons, the red-shifted channelrhodopsin variant VChR1 was co-expressed with a newly developed optogenetic pH reporter pH-Tomato targeted to the vesicular lumen as a synaptophysin fusion (SypHTomato). The authors used a single beam of green light (~560 nm) to both stimulate the actuator and excite the reporter. However, in contrast to the previous example, the authors used frequency modulation rather than intensity/wavelength modulation to achieve reporter imaging without further actuator stimulation. Specifically, the authors initially used a low acquisition frequency (requiring a low frequency of pulsed illumination) to image SypHTomato without causing substantial VChR1 activation. To activate sufficient VChR1 molecules for triggering a physiological response, they simply increased the rate of pulsed illumination, which allows a sufficiently large number of activated VChR1 molecules to accumulate. An apparent advantage of this approach is its relative simplicity, as no custom instrumentation or sophisticated optical setup is required.

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In the two examples (Guo et al., 2009; Li and Tsien, 2012) described above, the authors solved the problem of spectral overlap by taking advantage of differences in light levels (intensity of continuous illumination or frequency of periodic illumination) required to activate the optogenetic actuator (higher intensity and higher frequency) and to excite the optogenetic reporter (lower intensity and lower frequency). Although these solutions are clearly effective in these cases, they are not ideal. The ideal solution is to have actuators and reporters that can each be activated or excited with a wavelength of light that is not absorbed by the other optogenetic tool. In the following sections, we discuss a variety of additional practical strategies that can, or likely soon will, provide nearly ideal solutions to this problem.

New colours of optogenetic reporters

New optogenetic reporters based on far-red or near-infrared fluorophores are attractive candidates for minimising spectral overlap with optogenetic actuators activated with blue or green light. Numerous efforts had been made to shift the excitation and emission spectra of FPs towards the far-red range. One of the earliest examples of this was the engineering of the DsRed-derived mPlum variant with excitation and emission maxima at approximately 590 and 649 nm (Wang et al., 2004). Later efforts pushed the emission maxima to ever-longer wavelengths while also improving the brightness relative to mPlum, which suffered from a relative low quantum yield of 0.10. Recent examples of brighter and further red shifted far-red FP include mNeptune (excitation and emission maxima at ~600 and ~650 nm) (Lin et al., 2009) which is derived from mKate (Shcherbo et al., 2007); TagRFP657 (excitation and emission maxima at ~611 and ~657 nm) (Morozova et al., 2010) which displays better photostability, pH stability and less residual green fluorescence; E2-Crimson (excitation and emission maxima at ~611 and ~646 nm) (Strack et al., 2009) with fast maturation and decreased cytotoxicity and eqRFP670 with the most red-shifted emission (excitation and emission maxima at ~605 and ~670 nm) (Shcherbo et al., 2010) reported to date. Clearly, this ever-growing class of far-red FPs provides new opportunities for creating new optogenetic reporters with minimal spectral overlap with many of the optogenetic actuators. It is expected that the coming years will see many exciting developments in this direction.

An alternative approach to developing new genetically encoded fluorophores has been to abandon FP-homologues and turn to engineered bacteriophytochrome proteins (Shu et al., 2009; Filonov et al., 2011). Bacteriophytochromes are bacterial photosensory receptors that absorb near-infrared light due to a bound biliverdin chromophore, a ubiquitous product of heme catabolism. Bacteriophytochrome proteins from both *Deinococcus radiodurans* (Shu et al., 2009) and *Rhodopseudomonas palustris* (Filonov et al., 2011) have now been converted into so-called infrared FPs with excitation and emission maxima at approximately 690 and 710 nm, respectively. A major obstacle for using bacteriophytochromes as substitutes for GFP-type FPs in living animals is the uncertainty in the availability of biliverdin and their dimness (quantum yield less than 0.08). In principle, most of the engineering strategies applied to make FP-based reporters could be applied to bacteriophytochrome proteins as well. These near-infrared optogenetic reporters, and future generations that exhibit brighter fluorescence, could be used in combination with essentially any of the existing optogenetic actuators with little to no spectral overlap.

2P excitation

As discussed in the previous two sections, there are now several examples of taking advantage of light intensity or illumination frequency to tackle the problem of spectral overlap between optogenetic actuators and reporters. A conceptually similar, yet experimentally distinct, approach to overcoming this problem is to exploit differences in 2P cross-section to achieve specific excitation or activation. In 2P excitation, two photons with half the energy (twice the wavelength) are simultaneously absorbed by a chromophore, promoting it to the same excited state as it would normally reach using standard 1-photon (1P) excitation. Importantly, 2P excitation spectra (more correctly referred to as 2P cross-sections) of many FPs are often not simple rescaled versions of their 1P excitation spectra but exhibit additional peaks (Drobizhev et al., 2011). These additional peaks provide opportunities for excitation at wavelengths that separate well from then activation or excitation spectra of a second optogenetic actuator or reporter. For instance, ChR2 has a 2P absorption cross-section maxima at approximately

920 nm (Rickgauer and Tank, 2009), so it can potentially be combined with reporters that have distinct 2P cross-sections. It has been shown that, by using 2P excitation at 810 nm (with intensities <30 mW), Ca^{2+} imaging with the synthetic dye-based reporters Fluo-5F and Alexa 594 can be achieved without causing activation of ChR2 (Zhang and Oertner, 2007). In principle, a similar experimental setup might be suitable for the combined use of ChR2 and green optogenetic Ca^{2+} reporters such as G-CaMP3 (Tian et al., 2009) or G-GECO (Zhao et al., 2011).

Bioluminescence resonance energy transfer

An interesting strategy for partially minimising the problem of spectral overlap between optogenetic actuators and reporters is to use self-illuminating reporters based on the phenomenon of bioluminescence resonance energy transfer (BRET). BRET is similar to FRET but with a bioluminescent protein (*e.g.*, *Renilla* luciferase) used in place of the FP donor (Xu et al., 1999). As in FRET, excited-state energy is passed to an acceptor FP (typically YFP or GFP) in a distance-dependent manner, such that the modulation of the distance changes the efficiency of the process and the hue of the emitted light (Pfleger and Eidne, 2006; Xia and Rao, 2009). As no excitation light is necessary for the reporter, this approach completely avoids the problem of unwanted actuator excitation when imaging the reporter. However, the fact that the BRET acceptor could be excited by the light used to activate the actuator remains potentially problematic.

BRET-based optogenetic reporters have now been used for many live-cell applications, such as monitoring oligomerisation of G-protein coupled receptors (Kocan et al., 2008; Hollins et al., 2009) and transcription factor oligomerisation (Germain-Desprez et al., 2003). Luminescent analogues of the cameleon-type Ca^{2+} reporters have also been described (Saito et al., 2010). A major drawback of BRET is the lower photonic flux (23 photons/s for the best luciferases versus thousands of photons/s for a typical FP), which leads to longer integration times being required to achieve satisfactory signal-to-noise (Wu et al., 2009).

Although not based on BRET, an alternative application of bioluminescence are optogenetic reporters of protease activity which are based on a single circularly permuted luciferase that dramatically increases the efficiency of substrate oxidation upon cleavage by the protease of interest (Kanno et al., 2007; Gammon et al., 2009). Despite the advantage of not requiring excitation light, the utilisation of FRET and other FP-based reporter strategies remains far more common than the usage of BRET.

Summary and outlook

Genetically encoded reporters and genetically encoded actuators comprise the two main compartments of the optogenetic toolbox. In this review, we aimed to systematise the established transduction mechanisms of these engineered proteins and outline some of the challenges that need to be overcome to move this field of research forward. We believe that the information compiled in this review will not only be of interest to those that develop new reporters with improved features and new specificities, but also to the many cell biologists that routinely use these tools in their experiments. Many of the probes that we mentioned as examples leave space for further improvement and we therefore did not indicate 'best choices'. Rather, we would advise probe 'end users' to test a few different variants for each type of probe. This approach will not only increase the chances of success but will also help to generate enough experience so that 'best choices' crystallise in future.

Conflict of interest statement

The authors have declared no conflict of interest.

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