

Fluorescent proteins: a cell biologist's user guide

Erik Lee Snapp

Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA

Fluorescent Proteins (FPs) have revolutionized cell biology. The value of labeling and visualizing proteins in living cells is evident from the thousands of publications since the cloning of Green Fluorescent Protein (GFP). Biologists have been flooded with a cornucopia of FPs; however, the FP toolbox has not necessarily been optimized for cell biologists. Common FP plasmids are suboptimal for the construction of proteins fused to FP. More problematic are commercial and investigator-constructed FP-fusion proteins that disrupt important cellular targeting information. Even when cell biologists correctly construct FP-fusion proteins, it is rarely self-evident which FP should be used. Important FP information, such as oligomer formation or photostability, is often obscure or anecdotal. This brief guide is offered to assist the biologist to exploit FPs in the analysis of cellular processes.

Introduction: why an FP user's guide?

Hundreds of reviews, books, methods chapters and websites are devoted to FP technology, selecting FPs for their physical features, and describing an ever-expanding list of applications for FPs [1–11] (<http://micro.magnet.fsu.edu/primer/techniques/fluorescence/fluorescentproteins/fluorescentproteinshome.html>). GFP and other FPs have become pervasive in modern biological sciences and were recently recognized with the 2008 Nobel Prize in Chemistry. Despite the ubiquity of GFP, its use does not conform to a standardized one-size-fits-all protocol. Some companies, such as Clontech (Invitrogen), offer a user's manual for FPs. However, much of the information provided concerns the FPs themselves, with little information concerning FPs in the context of fusion proteins within cells, one of the most popular FP applications. I have prepared this guide for cell biology applications of FPs to assist novices and expert users alike in good practice and to help to avoid costly mistakes.

GFP and the other FPs are all inherently fluorescent proteins. Osamu Shimomura was able to purify GFP from the jellyfish *Aequorea victoria* and demonstrated that the protein emitted bright green fluorescence when illuminated with ultraviolet light (Figure 1A) [12]. For several years it remained unknown whether the protein required additional jellyfish factors to become fluorescent or whether the protein could autocatalyze the formation of the fluorophore. Doug

Prasher successfully cloned the GFP gene from jellyfish in 1992 [13] and hypothesized that GFP could form a fluorescent molecule in a heterologous environment. Martin Chalfie obtained the clone from Prasher, expressed it in *E. coli* [6], and confirmed that GFP could fluoresce even if expressed in an organism from a different biological kingdom. This finding ushered in a new era in cell biology where proteins of interest could be visualized with genetically encoded optical tags in live cells (Figure 1B) or even in whole animals. Work in the laboratories of Roger Tsien, Atsushi Miyawaki, Konstantin Lukyanov, and many others has led to a deep understanding of GFP structure (Figure 1C) and the mechanism of fluorescence, leading to enhanced GFP (EGFP, with improved fluorescence and expression properties) as well as to dozens of FPs of different colors (Figure 1D) or with unusual properties such as photo-activation [4]. Armed with a toolbox of powerful reagents and modern microscopes, biologists can now follow the spatial and temporal dynamics of cells, organelles, and individual proteins at high resolution.

Features of FP expression plasmids

When purchasing or requesting an FP plasmid one is typically asked to select the 'N' or 'C' version. These terms refer to the original Clontech (Invitrogen) EGFP plasmids and indicate the position of the multicloning site relative to the FP. N constructs place the protein of interest at the N-terminus of an FP while C places the protein at the C-terminus of an FP. For ease of subcloning into other FP plasmids, nearly all FP cDNAs have been integrated into these plasmids. The N and C plasmids contain a resistance marker suitable for both bacterial selection and the generation of stable mammalian cell lines (kanamycin for bacteria and G418 for mammalian cells). Both plasmids utilize one of the strongest promoters available (from cytomegalovirus), and though this allows the production of robust levels of FP or FP-fusion proteins, it will probably over-express most cellular proteins substantially. The N and C plasmids are excellent for simply expressing an untagged FP but have issues for the construction of some fusion proteins (see below).

Biophysical and biochemical properties of FPs

Currently there are dozens of FP options available when designing an experiment. Which one to select? The answer

Corresponding author: Snapp, E.L. (esnapp@aecom.yu.edu)

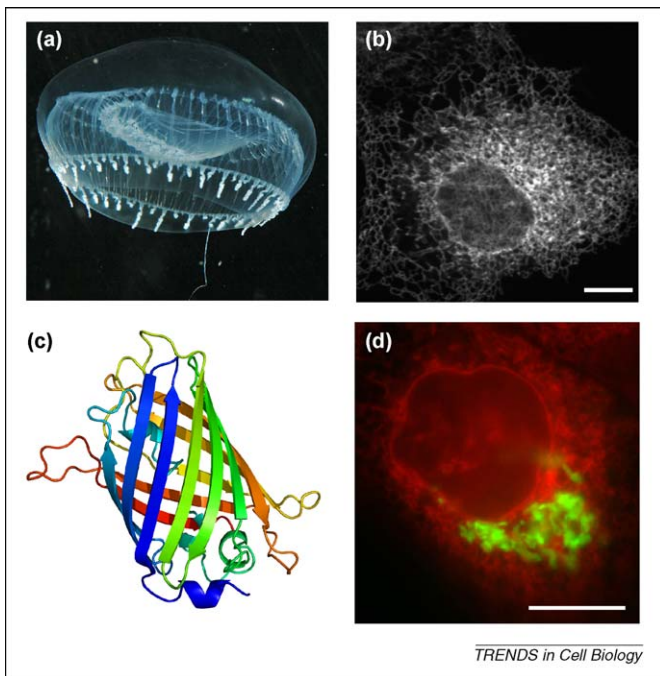


Figure 1. GFP from jellyfish to expression in mammalian cells. (a) The jellyfish *Aequorea victoria*. Image provided by Sierra Blakely. (b) GFP targeted to the endoplasmic reticulum of a mammalian fibroblast. (c) Ribbon diagram of the β -barrel structure typical of FPs. Image produced by Richard Wheeler from PDB:1EMA rendered in PyMOL. (d) Co-expression of an endoplasmic reticulum targeted red fluorescent protein and a Golgi complex targeted GFP in a mammalian fibroblast. Scale bars = 10 μ m.

can change every few months as improved FPs are reported in the literature, though newer does not always equal better. Whichever FPs one is considering, some key features are fundamental for any FP experiment. Spectral and biochemical properties are important for FPs and these are usually provided either in the original paper describing the FP or in an accompanying data sheet (see Box 1). With few exceptions, investigators need the brightest, most photostable, least phototoxic, and fastest-folding

FP to achieve robust fluorescent signals. For FP-fusion proteins, our lab primarily uses the FPs listed in the first part of Table 1. Superfolder GFP is showing great promise for fusion-protein constructs that appear comparatively dim, probably because the fusion proteins interfere with FP folding (see Ref. [14], in particular Figure 4). That is, just as FPs can affect the functionality of a fusion protein (see following sections), a protein of interest can disrupt FP folding and affect the FP fluorescence signal.

FP codon optimization and sequence suitability

The majority of FPs have been developed from jellyfish and coral proteins. One major difference between these animals and mammals is the choice of amino acid codons. In 1996 Brian Seed and colleagues [15] improved the expression and fluorescence signal of GFP in mammalian cells by 40–120 fold by heavily modifying the GFP codon sequence to reflect mammalian codon preferences. Most commercially available plasmids have been codon-optimized for mammalian cells. However, some of the older FP plasmids lurking in lab freezers may not have been optimized and one GFP may not be equivalent to another. Mammalian codon-optimized FPs are not necessarily optimized for other model organisms such as *Drosophila* or yeast. Investigators working in non-mammalian systems should consider synthesizing codon-optimized variants of FPs (currently costing about \$350 for the average FP) for their model organism.

Even if an FP is codon-optimized, it is not necessarily suitable for some cellular environments. For example, TagRFP and mKO contain multiple cysteines and consensus N-glycosylation sites (N-X-S/T, where X is any amino acid except proline) that could modify the folding, size, and oligomerization of these FPs if targeted to the secretory pathway of eukaryotic cells [16]. Even EGFP and its variants contain two cysteines, and these can lead to disulfide-bonded oligomers in the endoplasmic reticulum (ER) [17]. Under more extreme conditions EGFP cannot fold correctly or fluoresce in the highly oxidizing environment of the

Box 1. Fluorescent protein spectral properties

The spectral properties of FPs determine whether an FP can be used with a particular imaging apparatus and whether other FPs can be used in the same cell or experiment. These properties include:

Absorbance: the wavelengths of light needed to excite a fluorophore.

Emission: the wavelengths of light produced by the excited fluorophore. The absorbance and emission spectra can be quite broad, and this will impact on the imaging setup and whether an FP can be combined with other fluorophores. Therefore, it is essential to know the full spectra of the FPs and the properties of the instrument to be used. Many core facilities have this information. If filter information is not available in a lab manual or in the microscope software, the information can be found on the fluorescence filters themselves. To access your fluorescence filters, refer to the user's manual, extract the fluorescence filter cubes: the information is on the filter cubes (see <http://www.olympusmicro.com/primer/techniques/fluorescence/filters.html>). With the instrument filter spectra one can determine whether the correct excitation light sources and emission filters are available for FP experiments.

Brightness: the product of quantum yield and extinction coefficient. This provides a useful reference as to whether an FP will be sufficiently bright for an experiment. Brightness of FPs can be compared to EGFP (30,000 $M^{-1}cm^{-1}$) or spectrally related FPs. The

practical consequence is that to study a protein expressed at low levels (i.e. most kinases and transcription factors) requires the brightest possible FP, whereas an abundant protein (e.g. tubulin, actin, GAPDH, chaperones) may permit use of a dimmer FP with more optimal spectral characteristics.

Brightness is reported only for the completely folded protein. Therefore, in cells, the rate of maturation of an FP may be as important as FP brightness. Maturation can range from minutes to hours and is often described in the relevant publications. However, the methods for determining and reporting maturation vary. One should determine whether the reported rate refers to immature nascent proteins that have not yet formed chromophores, or to mature proteins that have been denatured and then timed for the reappearance of fluorescence after removal of the denaturing conditions. The latter values are primarily for *in vitro* studies, as FP refolding is not a general concern in cells. Another caveat concerning maturation rates is that several studies report maturation under low-oxygen conditions. In cell culture, oxygen is more abundant and FP maturation rates are much faster.

Photostability: defines how long a population of FPs can be continuously excited before photobleaching or destruction of the fluorophore. This value is often provided for arc lamp and laser excitation. In general, select FPs with high photostability (longer half-lives) to enable prolonged imaging of cells.

Table 1. Popular FPs and examples of successful FP-fusion proteins

Protein	Reference	Notes
FPs		
TagBFP	[37]	New bright photostable blue FP
Cerulean	[38]	Improved form of the cyan FP (CFP)
Monomeric EGFP	[1,15,20,21]	The original optimized green FP and best characterized FP for FP-fusion protein design
Venus or Citrine	[39,40]	Improved forms of yellow FP (YFP)
mCherry or mKate2	[3,22]	Popular red FPs
PA-GFP and PA-mCherry	[41,42]	Photoactivatable FPs
FP-Fusion Proteins		
GRP94-GFP	[43]	Luminal ER Chaperone
GFP-NMDAR1	[44]	Ion channel.
GFP-Tub1	[45]	Yeast α -tubulin
GFP-clathrin	[46]	Secretory pathway coat protein

periplasmic space of gram-negative bacteria [18]. In contrast, the cysteine-less mCherry readily folds in the same environment [19]. Therefore, FP amino acid sequences should always be examined carefully for potentially environment-sensitive sequences.

FP oligomeric state

Many FPs have a tendency to oligomerize either as part of their inherent structure (e.g. DsRed is an obligate tetramer) or when present in high concentrations on membranes or in oligomeric proteins (e.g. EGFP). It is therefore important to determine whether an FP is monomeric and whether this matters for the experiment. While FP oligomerization has become more commonly reported, the propensity of an FP to oligomerize is often unknown because oligomerization assays are not always robust or quantitative. Many papers describe an FP as monomeric without directly demonstrating monomerization or without reporting a K_d value. This point is not merely academic. Given that it can be difficult or expensive (up to \$500 per plasmid) to obtain an FP plasmid, it would be disturbing if the expensive FP is then found to oligomerize with the selected FRET biosensor or integral membrane fusion-protein. Currently, there are no accepted standards for how monomeric an FP needs to be for cell applications. Some researchers fuse new FPs to tubulin or actin to determine whether cytoskeletal structures form correctly. However, such assays overlook the effects of EGFP dimerization under other physiological conditions (see below). Therefore, investigators must confirm that an FP-tagged protein behaves similarly to untagged proteins in assays and environments relevant to the protein of interest.

FP oligomerization matters because FPs considered to be monomeric can form dimers at sufficiently high concentrations in cells. For example, EGFP forms dimers when fused to integral membrane proteins or is incorporated into oligomeric proteins [20]. As a consequence, fusion FPs can form inappropriate interactions leading to false-positive FRET signals [20] or distortion of cellular organelles [21]. For fusion proteins, the FP must be truly monomeric. Fortunately, EGFP and variants (CFP and YFP) can be monomerized with a single point mutation (A206K) [20,21].

FP applications in cells

Free FPs to mark cells

FPs can be expressed as free proteins either constitutively or under the control of an appropriately regulated promo-

ter. There are few restrictions on the choice of FP for these experiments other than the FP must be sufficiently bright. Tandem dimer FPs such as tdKatushka2 and tdTomato are excellent choices because they have two copies of an FP; this makes them exceptionally bright [22]. If FPs are being used as reporters of promoter activity, then chromophore formation time may be a consideration. Fast-folding FPs, such as mCherry and Venus, will rapidly report promoter activity. Note that such FP reporters offer little insight into message stability and generally reflect both cumulative promoter activity and the stability of the fluorescent protein because fluorescent proteins typically have half-lives of ~24 h [23]. Several groups have attached protease-degrons to FPs to enhance FP turnover and protein half-lives of ~2 h have been achieved [23]. Alternatively, another class of FPs – fluorescent ‘timers’ – change color with age and provide relative measures of ratios of recently synthesized and old FPs [24,25].

FP-fusion proteins

The ability to visualize a protein’s distribution and dynamics in a subcellular compartment has opened new opportunities in cell biology [26–28]. Correct design and characterization of FP-fusion proteins are essential for the interpretation of FP-fusion protein studies.

Some investigators take short cuts and ‘clone by phone’, but while it is tempting to rely on others to create FP-fusion proteins of interest there are important reasons for making your own constructs. One must be skeptical of any constructs received from other labs or companies. Not all constructs are made with consideration of protein-targeting domains (see below). Also, many FPs are incorrectly labeled. For example, a ‘DsRed construct’ could be either the monomeric or the tetrameric form. The personal experience of the author affords another example. Photobleaching experiments to study the protein dynamics of GFP-fusion proteins require that the FP photobleaches irreversibly. On one occasion it was found that GFP-fusion proteins from a collaborator produced unexpectedly rapid protein mobilities in cells. Sequencing revealed that the GFP contained the three EGFP mutations and two additional mutations reported to enhance brightness. Control experiments revealed that this GFP, unlike standard EGFP, underwent nearly 80% reversible photobleaching (also termed photoswitching) (our unpublished results; see also studies in Refs. [16,29,30]). Not all ‘EGFPs’ are equal! Whenever obtaining an FP construct from another lab,

Table 2. Eukaryotic protein targeting domains with position requirements

Sequence Position	Localization	Notes
N-terminal domains		
Signal sequence	ER	Usually post-translationally cleaved
Presequence	Mitochondrion	Amphipathic helix that is usually post-translationally cleaved
Myristoylation sequence	Cytoplasmic face of cellular membranes	Initiating methionine is cleaved
C-terminal domains		
-KDEL	ER retrieval motif for luminal proteins	Domain must be in the ER lumen.
-KKXX (X is any amino acid)	ER retrieval motif for integral membrane proteins	Domain must be exposed in the cytoplasm
-SKL	Peroxisome lumen	
-GPI anchor sequence	Binds luminal and extracellular leaflets of cellular membranes	A fragment at the C-terminus of the protein is cleaved for fusion with GPI
-Tail anchor	ER or mitochondrial membrane	
-CAAX (X is any amino acid)	Palmitoylation	

politely request a plasmid map and a sequence file. If a sequence file is not available, sequence the FP construct before performing any experiments. Avoid working with mystery reagents! This anecdote also illustrates the importance of collecting stable baseline values for time-resolved fluorescence experiments to help identify phenomena such as photoswitching. Finally, unusual photophysical properties of FPs are not always problematic and can be exploited to develop new imaging techniques. For example, photoswitching plays an important role in the super-resolution technique of PALM (ref. [31]; see also the article by Jennifer Lippincott-Schwartz in this issue).

Why GFP has not made antibodies obsolete

Whenever an epitope tag (*any* epitope tag: EGFP, myc, His, HA, and so forth) is added to a protein the tag can modify protein function either by sterically blocking protein interactions with substrates or by disrupting targeting sequences (see next section). Knowledge of the protein and engineering of the epitope tag to avoid disruption of protein function or targeting can circumvent such issues.

An antibody against the native protein is a key reagent for epitope-tagging experiments. An antibody can confirm that the tagged protein (i) localizes correctly by immunofluorescence, (ii) is the correct size and expressed at levels similar to the untagged protein in an immunoblot, and (iii) interacts with known substrates as demonstrated by co-immunoprecipitation. Simply tagging a protein with an FP to avoid having to make an antibody will not address all of these important points. Any FP-fusion localization or related information must be independently verified with an antibody to confirm that the FP has not disrupted protein behavior or localization.

In addition to an antibody, fusion protein studies require that a functional assay is available. The importance of a functional assay cannot be overstated. Even if the tagged protein localizes correctly in a cell, it is crucial to confirm that the tagged protein behaves as the native protein. The objective of adding an FP to a protein of interest is to monitor the localization and dynamics of that protein in cells. A nonfunctional FP-tagged protein will be uninformative at best, and most likely will be misleading. Some examples of FP-tagged proteins with demonstrated functionality are listed in the second part of Table 1.

Targeting sequences and where to insert the FP

Once a bright monomeric FP has been selected, a functional assay for the protein of interest has been established, and a good antibody against the native protein has been obtained, it is now necessary to decide where to place the FP. Significant knowledge of the protein of interest is essential to successful FP-fusion design, and care should be taken to ensure that FP-fusion does not block the normal localization and functionality of the protein of interest. A crucial factor in FP placement involves knowledge of the different types of protein motifs for targeting, retrieval, and retention, as well as of the contextual and positional requirements of the motifs.

Many cellular proteins reside within organelles or sub-compartments. Protein localization depends on information encoded within the protein's primary sequence [32], and it cannot be emphasized enough that *protein targeting sequences generally depend on the context and position of the sequence within the protein*. Many protein-targeting sequences must be at the extreme N- or C-termini of the protein (see Table 2). For example, most secretory proteins will not enter the endoplasmic reticulum (ER) unless the signal sequence is positioned at the N-terminus of the protein. Similarly, proper localization of resident ER proteins requires that the ER retrieval motif (-KDEL or -KKXX) is at the absolute C-terminus of the protein so as to interact with the retrieval machinery. Thus, for example, placement of an FP before the signal sequence or after the ER retrieval motif will disrupt the localization of the FP-fusion protein. The positioning requirements of the localization sequences of a protein of interest will determine what sites are appropriate for FP fusion.

Approximately 20 percent (~6,000 genes) of the human genome encodes secretory proteins [33]. A further 1500 proteins localize to mitochondria, up to 8400 are transported into the nucleus, and 60 are targeted to peroxisomes. Cytoplasmic proteins can also contain position-dependent posttranslational modifications such as myristoylation and palmitoylation. Overall, at least one third of the genes in the human genome encode proteins with position-dependent information. The tagging of each protein with an FP (or *any* epitope tag) thus requires evaluation of the appropriate and inappropriate positions for FP insertion relative to the protein of interest. The large number of proteins with targeting information

Box 2. FP-fusion protein design

Optimal design of FP-fusion proteins requires significant knowledge of the protein of interest. FP tagging is not generally recommended for characterizing novel or poorly studied proteins. Instead, the investigator should have as much information about a protein as possible so as to ensure that the FP can be placed in the least perturbing location for the protein of interest. This is discussed in great detail elsewhere [36]. Briefly, FPs can be modified and placed before or after a relevant targeting sequence using standard molecular biology techniques. For example, a resident ER luminal protein could have the FP tag engineered into the coding sequence between the signal sequence and the mature protein, or between the mature protein and the KDEL ER-retention sequence (Figure 1A). We have used PCR amplification to generate FPs with a KDEL sequence at the extreme C-terminus. The full-length protein cDNA, minus the KDEL sequence, is then placed upstream of the FP (Figure 1B). To improve the accessibility of interacting proteins to the targeting domains, one can add a short linker domain of small hydrophilic amino acids, for instance 2–6 copies of alternating glycine and serine.

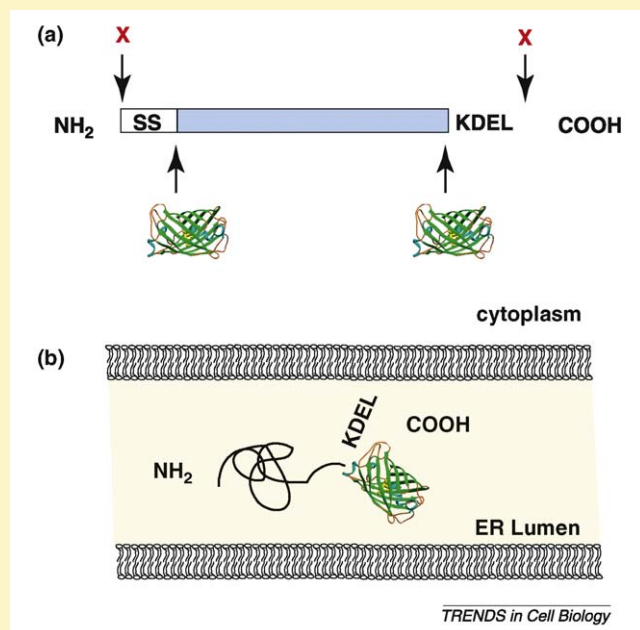


Figure 1. FP-fusion protein design. (a) Illustration of a typical resident ER luminal protein primary sequence. The signal sequence (SS) occurs at the extreme N-terminus and the KDEL ER retrieval motif occurs at the absolute C-terminus. Placement of an FP at either the N- or C-termini will disrupt essential position-dependent targeting information and is strongly discouraged (red Xs). In contrast, placement of the FP after the SS or before the KDEL will generate a protein correctly targeted to and retained in the ER lumen. (b) The resulting FP-fusion protein, when the FP is placed immediately before the KDEL sequence. Note that the N- and C-termini of GFP occur at the same end of the β -barrel structure.

suggests all potential fusion proteins should first be analyzed for targeting sequences.

It is curious that numerous publications, often in reputable journals, employ one-size-fits-all FP tagging strategies to follow the localization and behavior of proteins in cells. Although it is clearly attractive to develop high-throughput approaches to describe the latest ‘-ome,’ careful reading of the FP-tagging strategy may reveal serious issues with the approach and the associated data. Many protein-targeting sequences have stringent position requirements. Placement of an FP before or after a targeting sequence could mask the targeting sequence, so

disrupting the correct targeting of the protein, and thus makes indiscriminate GFP-tagging of proteins a dubious practice (Box 2). For example, some studies have engineered an FP before the start, or at the terminus, of all open reading frames. The former approach will prevent most secretory proteins from entering the ER, will stop mitochondrial proteins from translocating into the mitochondrion, and halt the addition of myristoyl groups. The latter approach will prevent both the retention of proteins in the ER and protein entry into peroxisomes. Thus, whole classes of proteins will be incorrectly targeted and processed. The resulting data are of questionable value. Despite such concerns, some companies now offer thousands of cDNAs fused to EGFP at either the N- or the C-terminus. To give an example, inspection of constructs derived from the luminal ER chaperone calreticulin revealed that the open reading frame contains both a signal sequence and a –KDEL retrieval motif, and yet both EGFP-fusion options were available even though neither would be physiologically functional. Hardly worth \$800! However, one excellent resource for obtaining a pre-constructed FP-fusion protein plasmid can be found at <http://www.addgene.org> – published FP-fusion constructs are available in a searchable database, have been well annotated, and are available for a modest fee of \$65 per plasmid.

It is not intended that this review should give the impression that every protein is a ‘minefield’ of targeting domains. Rather, position-dependent targeting domains are predominantly found at the N- and C-termini of the protein. This simplifies the analysis and generation of FP-fusion proteins. Bearing in mind the importance of FP position, numerous studies have successfully created FP-tagged proteins that retain the functionality of the wild-type untagged protein (Table 1). While the design of FP-fusion proteins (Box 2) requires significant knowledge of the protein of interest, targeting sequences are not always apparent in the primary sequence of the protein. Indeed, many of the sequences in Table 1 are not defined as absolute consensus sequences. This is because many targeting sequences have biochemically defined properties but lack a common primary sequence. For example, every secretory protein in the human genome has a unique signal sequence that ranges in size from 14–70 amino acids [34]. Web-based resources including GenBank, ExpASY, and SignalP 3.0 (<http://www.cbs.dtu.dk/services/SignalP/>) can assist in identifying signal sequences. Given these complexities, FP-tagging is not a recommended approach for characterizing novel or poorly studied proteins.

An additional consideration for FP-fusion design arises because most FP plasmids are in the form of the Clontech N vector. The N construct contains a strong mammalian Kozak sequence and an initiating methionine for the FP. This design is excellent for the expression of a given FP by itself, but can be suboptimal for fusion proteins because translation of the FP can take place, at least potentially, independently of the translation of the attached fusion protein sequence, due for instance to leaky ribosomal scanning (our unpublished data and Ref. [35]). To reduce the likelihood of such phenomena it is recommended that the FP sequence is first amplified without a methionine or Kozak sequence and then fused in frame with the cDNA for

the protein of interest. Once constructed, confirmation should be obtained of the FP-fusion protein sequence, functionality, localization relative to the untagged parent protein (by immunofluorescence), and fusion protein size (by immunoblotting). Only at this point is the investigator ready to unlock the full potential of FP-fusion proteins in living cells or even whole organisms.

The future FP guide: an updatable web-based user's guide

The pace of FP development has created the need for a centralized FP resource site on the internet. There are some FP-related sites but the information and tools they contain tend to be spread out over multiple websites and are often somewhat out-of-date. Instead, the user community needs to develop a freely accessible and searchable FP resource website that can be as easily updated as pages on the Wikipedia and Genbank sites. Users should be able to access both the nucleotide and amino acid sequences, the spectra and fluorescent properties of all FPs, as well as notes on their use, including the oligomeric state and pKa of an FP, and links to related older and newer FPs. An ideal site would also provide a widget for overlaying multiple FP spectra to aid in experimental design. Finally, a user comment section with matters arising for each FP could help to alert other users of FP applications and FP caveats. Given the success of GenBank, EXPASY, and other resource websites, an FP website should be feasible and would be of great utility to everyone developing and using FPs. With better organization and accessibility of FP information, the FP toolbox will be fully exploitable by all researchers.

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