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Design and Use of Fluorescent Fusion Proteins in Cell Biology

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The discovery that green fluorescent protein (GFP) variants and coral fluorescent proteins can be functionally expressed in heterogeneous systems has revolutionized cell biology (Lippincott-Schwartz et al., 2001; Miyawaki et al., 2003). Unmodified fluorescent proteins (FPs) can be visualized by fluorescence microscopy and can serve as probes of environments within living cells. The addition of targeting and retention sequences to FPs can be exploited to highlight specific cellular organelles and to follow their dynamics. The ability of FPs to fold, even when fused to cellular proteins, has made it possible to directly study the biology of proteins in vivo. A protein of interest can be monitored in cells or even in whole animals without having to purify, label, and deliver the protein into cells. Thus, it is now possible to label and observe proteins in previously inaccessible environments, such as organelle lumena. Fusion of FPs to proteins of interest can reveal a wealth of data, including information on a protein's steady-state distribution, dynamics, history, and association with other proteins (Lippincott-Schwartz et al., 2001; 2003). In this unit, strategies and background for designing and creating fluorescent fusion proteins (FFPs) are described.

To design an FFP, the investigator must consider what the FFP's intended use is, which fluorescent tag to add, whether the FP has complicating issues related to the protein of interest's environment (e.g., pH sensitivity, enhanced aggregation), and where to insert the FP. The actual construction of FFPs can be readily accomplished using standard molecular biology techniques and will only be described in general terms in the Basic Protocol. Investigators seeking specific advice on cloning techniques are referred to *Current Protocols in Molecular Biology* (Ausubel et al., 2005) or *Molecular Cloning: A Laboratory Manual* (Sambrook and Russell, 2001).

DESIGN OF A FLUORESCENT FUSION PROTEIN

Most fluorescent protein (FP) cDNAs are commercially available from BD Biosciences Clontech or from the laboratories that first described the FP. Often, FP coding regions are positioned adjacent to a multicloning site in the plasmid for ease of subcloning. The cDNA for the protein of interest can sometimes be directly cloned into the FP vector. However, in many cases, construction of a fluorescent fusion protein (FFP) will necessitate modification of the protein of interest and/or the FP. As described below, placement of an FP can affect the localization and functionality of the protein of interest. Because the required modifications will vary on a case-by-case basis, two different general cloning strategies are described below:

Strategy 1: When a protein's functional and targeting domains are unknown

Even if only minimal information on a protein is available, it is still possible to study the protein's environment and behavior in cells. However, the investigator should first assess the steady-state distribution of the unmodified protein of interest (e.g., by immunolocalization; *UNIT 4.3*). This is necessary to ensure that the corresponding FFPs localize properly. It is also useful to compare the distribution of the untagged protein in different cell types, as some proteins localize very differently in different types of cells. For example, procollagen only folds properly and exits from the ER in cell types expressing specific chaperones (Nagai et al., 2000).

To maximize the likelihood of creating a functional and properly targeted FFP, the investigator should design two constructs. One construct should contain the FP at the NH_2–terminus of the protein of interest, and the other should have the FP at the COOH-terminus, as many proteins fold with their NH_2– and COOH-termini exposed on the protein surface, rather than buried in the protein core (Hovmöller and Zhou, 2004). It is often useful to include a small linker of two to ten amino acids, such as glycine interspersed with serine residues (to enhance the solubility of the linker), to provide flexibility between the FP and the protein of interest (Miyawaki et al., 2003). The linker can help promote proper folding and functioning of both the FP and the protein of interest. Note that the necessary size of a linker can only be determined empirically. The position of the FP can also affect the need for a linker. For example, the COOH-terminus of enhanced green fluorescent protein (EGFP) is floppy and rarely requires a linker when fused to the NH_2–terminus of the protein of interest (Miyawaki et al., 2003). A linker can be added by PCR to the cDNA encoding the protein of interest or to the FP cDNA.

The final FFP construct will contain an in-frame fusion between the FP and the protein of interest, an unambiguous initiating methionine within the appropriate Kozak sequence (for eukaryotes, this is 5′-ACCATGG-3′, where the internal ATG is the initiating methionine; Kozak, 1992), a linker between the FP and the protein of interest (if necessary), and appropriate regulatory elements (e.g., promoter, 5′ and 3′ untranslated regions). Appropriate regulatory elements are often included in commercially available FP subcloning vectors.

Once a cloning strategy and PCR primers have been designed, the investigator can generate the FFP construct. The final step of a cloning strategy is to place the FFP in an appropriate expression vector. For example, the cytomegalovirus promoter–containing EGFP-C1 vector (Clontech) can be used to express the FFP in mammalian cells, but not in bacteria or yeast. Insertion of the construct into an appropriate vector can be accomplished by ligation into a multicloning site.

In preparation for future experiments, it is often useful to make several color variants of the desired FFP. Because the GFP variant sequences are identical to each other at the NH_2– and COOH-termini, it is easy to use the same PCR primers to amplify multiple variants simultaneously.

Strategy 2: When a protein's function can be assayed and targeting domains have been identified

In this situation, the investigator can exploit the full potential of FFPs. The FP can be inserted at an optimal position (i.e., one at which the FP will not interfere with targeting domains or protein folding), and the resulting FFP can be assayed for function. As in strategy 1, it is important to be able to assess the steady-state distribution of the wild-type protein of interest. This will help the investigator distinguish whether the FP affects the spatial distribution of the protein being studied.

The example of a lumenal endoplasmic reticulum (ER) protein illustrates some of the considerations affecting FP placement (Fig. 21.4.1). A typical lumenal ER protein contains two critical sequences with targeting information—a signal sequence at the NH_2–terminus, and an ER-retention sequence at the COOH-terminus. The signal sequence is essential for the targeting and translocation of the nascent peptide into the lumen of the ER (Martogolio and Dobberstein, 1998). Thus, the FP must be placed after the signal sequence, but where?

If the functional domain of the protein is near the COOH-terminus, then placing the FP after the signal sequence is a reasonable strategy. It is advantageous to place the FP two to ten amino acids downstream of the predicted signal-sequence cleavage site (R.S. Hegde, unpub. observ.).

This will enhance the efficiency of signal-sequence cleavage and help promote the efficiency of translocation of the FFP into the ER.

If the functional domain of the protein is toward the NH_2–terminus, then the FP should be placed toward the COOH-terminus, but not necessarily at the absolute terminus. For example, in animal cells, the KDEL motif must account for the final four amino acids of the protein, as this motif functions as a lumenal ER retention sequence (Pelham, 1990). In such a case, the FP should be inserted just before the start of the KDEL motif. Additional examples of appropriate and inappropriate FP placement sites are illustrated in Figure 21.4.1. Once the FP has been inserted, as in strategy 1, the last step is to place the FFP in an appropriate expression vector.

Finally, in both strategies, the investigator must confirm that the FFP construct has the correct DNA sequence and assess whether the expressed FFP is fluorescent, localizes in a pattern similar to that of the wild-type protein within the cell (e.g., by performing immunofluorescence and colocalization studies; *UNIT 4.3*), and retains the functionality of the native protein (as determined by the investigator's own assays). In the absence of functional characterization, the investigator should be vigilantly skeptical of inferring too much from studies of FFPs.

COMMENTARY

Background Information

Considerations for the design and application of FFPs—The various fluorescent proteins (FPs) have different advantages and limitations. Before designing and generating an FFP, the investigator needs to identify what questions the FFP will be used to address. It is worth spending some time asking whether the planned construct will be able to fulfill the stated purpose. Additional concerns include whether the FFP will be sufficiently bright, whether it will be appropriate for the time scale of the planned experiment, and whether the necessary equipment is available for the application.

The simplest fluorescent fusion proteins (FFPs) are FPs fused to targeting sequences (e.g., a nuclear localization sequence or a signal sequence) for the highlighting of an organelle or a cellular domain of interest. Such FFPs permit the investigator to colocalize a protein of interest with a specific organelle or to follow the dynamics of an organelle in a living cell (Lippincott-Schwartz et al., 2001; Miyawaki et al., 2003). In addition, these FFPs can be coupled with photobleaching methods (*UNIT 21.1*) to probe the viscosity or crowdedness of a cellular environment (Dayel et al., 2000; Nehls et al., 2000; Lippincott-Schwartz et al., 2001). FFPs containing a full-length protein fused to an FP are potentially valuable tools that can be exploited to illuminate a protein's function or behavior in its native environment.

How much of a fluorescent protein is enough?—The first issue to carefully consider is the normal expression levels of the protein of interest. Ranges of expression levels for a variety of proteins are provided in Table 21.4.1. Compare these values with the concentrations of FP that are required for visualization over background fluorescence in cells. For example, to achieve a twofold increase in fluorescence over background fluorescence, enhanced green fluorescent protein (EGFP) must be expressed at 200 nM (Patterson et al., 1997). Thus, to visualize a homogeneously distributed cytoplasmic FFP, the FFP must be expressed at levels two orders of magnitude higher than those for a kinase such as MAPKKK (Table 21.4.1). However, if all of the FFP will be concentrated in discrete compartments or domains, then low expression levels may be sufficient to visualize the protein or organelle of interest; in contrast, if a protein normally binds to a receptor present at low levels, the excess fluorescence of the unbound FFP could obscure the physiologically relevant population. More importantly, the potential biological consequences of the overexpressed protein should not be underestimated.

Therefore, knowing the normal expression levels and localization of one's protein of interest can greatly assist in the planning and interpretation of experiments. To determine a protein's relative expression level, the investigator can compare immunoblots from lysates of cells that natively express the protein of interest and lysates of cells stably transfected with the FFP, using the same antibody for the native protein and the FFP (*UNIT 6.2*).

Rate of fluorophore maturation—FFPs are generally useful as markers for and probes of protein and organelle dynamics. However, the temporal aspects of FP maturation may place constraints on the utility of FFPs in certain experiments. For example, current FFPs are unlikely to be useful for following the behavior of nascent proteins within the first few minutes of translation. This is because EGFP takes up to 30 min to fold and fluoresce in solution (Heim et al., 1994). However, EGFP may become fluorescent more rapidly in cells (Prendergast, 1999).

The *Discosoma* red fluorescent protein (DsRed) is even slower, taking up to 48 hr to make 90% of all newly synthesized proteins fluoresce (Baird et al., 2000). However, new red FP variants are available that fold in <1 hr (Bevis and Glick, 2002; Campbell et al., 2002). The slow folding of one DsRed variant, DsRed-E5, has been exploited to visually distinguish between proteins younger and older than 9 hr (Terskikh et al., 2000).

Size matters—Unlike an epitope tag, FP fusions are not inherently small modifications to proteins (Fig. 21.4.2). An FP represents a significant addition to a protein and thus may have steric consequences for protein folding, function, or targeting. GFP variants and the coral FPs are 27 kDa in size. The crystal structures of all of the FPs show β-barrels that are 3 nm in diameter and 4 nm in length (Yang et al., 1996). In addition, many of the coral FPs exist as obligate tetramers of these β-barrels (Baird et al., 2000), which not only increases their size but also tends to induce formation of aggregates. For these reasons, investigators need to be alert to FP-related effects on a protein's behavior. Alternatives to FPs are briefly discussed in the following section.

Selecting a fluorescent protein—Once the investigator has determined that an FFP will meet the requirements of the experiment, he or she must choose an FP. In the past few years, the variety of available FPs has increased dramatically. In addition to GFP and its spectral variants, coral FPs that span the visible spectrum and FPs with new functionalities have become available (Miyawaki et al., 2003). Many of the currently available FPs are listed in Table 21.4.2. Generally, the investigator will want to select the brightest possible FPs to increase the signal-to-noise ratio. The relative brightness values of various FPs are indicated in Table 21.4.2. It is also important to confirm that the necessary equipment (e.g., filter sets, excitation lasers) for using the FP of interest is available. BD Biosciences provides a useful Web resource that profiles the excitation and emission spectra of several fluorescent dyes and proteins (http://www.bdbiosciences.com/spectra/). Families of FPs that are currently available are described below. It is likely that improved variants will continue to become available, and so the investigator should regularly search the literature for the latest FPs.

Note that many of the FP cDNAs are either native sequences or have been codon-optimized for the intended host cell type. Codon-optimized variants are available for mammalian, fungal, and plant cells (Yang et al., 1996; Davis and Vierstra, 1998; Sheff and Thorn, 2004). If the FP expresses poorly in organisms such as bacteria or fungi, codon bias or Kozak sequence–related issues should be considered.

GFP and its spectral variants: The first FP to be cloned and characterized was green fluorescent protein (GFP) from the jellyfish species *Aequorea victoria* (Prasher et al., 1992). GFP folds to form a β-barrel bearing a triplet of internal amino acids (SYG) that

autocatalytically form a fluorophore within the barrel (Heim et al., 1994). Wild-type GFP does not fold efficiently, is sensitive to pH, and can undergo reversible photobleaching (Patterson et al., 1997), which is a problem in FRAP (fluorescence recovery after photobleaching) and FLIP (fluorescence loss in photobleaching) experiments (*UNIT 21.1*). To improve the utility of GFP, the codon bias was modified to make the protein more suitable for expression in mammalian cells, and two amino acids were mutated to enhance brightness and protein folding (S65T and P64L). The resulting variant is termed EGFP and is available from Clontech (Yang et al., 1996).

Note that EGFP and its variants have a modified amino acid numbering system. When EGFP was first created, amino acid 2, a valine, was deleted. However, publications generally use the original wild-type GFP amino acid numbering scheme. Thus, the fourth amino acid (starting from the NH_2–terminus) in EGFP would be numbered as amino acid 5 in most publications. This can be important when sequencing or mutagenizing the EGFP variants.

Point mutations can alter the spectral properties of EGFP to create blue (ECFP) and yellow (EYFP) variants, also available from Clontech. These variants differ in other ways besides spectral excitation and emission. Forexample, ECFP is substantially dimmer than EGFP, while EYFP is slightly brighter (Table 21.4.2). To calculate the brightness of an FP, multiply the protein's extinction coefficient (ε, the efficiency of photon absorption) by its quantum efficiency (ϕ, the ratio of photons emitted to photons absorbed; Table 21.4.2).

Recently, EGFP and its variants have been further improved. All EGFP variants can undergo weak transient dimerization in living cells (Zacharias et al., 2002). This dimerization can produce false-positive FRET results and lead to the reorganization of membrane structures when an enhanced FP is fused to a membrane protein (Fig. 21.4.3A; Zacharias et al., 2002; Snapp et al., 2003). Fortunately, dimerization can be disrupted by any one of three different point mutations (A206K, L221K, or F223R) without changing the fluorescent properties of EGFP (Zacharias et al., 2002). Such monomerized variants can be generated by mutagenesis or can be obtained from the author of this unit.

Another advance in EGFP variants has been the generation of brighter versions that fold more efficiently. Two YFP variants (called Venus and Citrine; Nagai et al., 2002; Griesbeck et al., 2001) that are more resistant to the effects of chloride ions and pH, as well as a CFP variant (called Cerulean Blue; Rizzo et al., 2004) that has improved fluorescence properties, have become available, and the reader is encouraged to use these improved versions.

Other groups have modified GFP to create environmental sensors. For example, the sensitivity of wild-type GFP to pH has been exploited by Miesenbock et al. (1998) to create mutants that can function as pH sensors.

Coral fluorescent proteins: GFP and its variants cover the visible spectrum from 400 to ~540 nm. Until recently, FPs were not generally available at the red end of the spectrum. FPs that emit at longer wavelengths are highly desirable, because autofluorescence is reduced at these wavelengths (Miyawaki et al., 2003). The discovery of a series of red FPs and chromophores in coral species remedied this deficit. The first of the coral FPs, DsRed, has a spectral emission in the range of 583 nm (Table 21.4.2). This property offers the investigator an additional marker for imaging in the same cell with CFP and GFP/YFP fusion proteins. However, wild-type DsRed, like wild-type GFP, has significant drawbacks. It is an obligate tetramer, it matures slowly (48 hr), and its excitation and emission profiles are in the same spectral range as EGFP during the first 6 hr of the maturation phase (Baird et al., 2000). Two enhanced versions of DsRed have become available—DsRed.T4 (Bevis and Glick, 2002) and mRFP (Campbell et al., 2002). DsRed.T4 matures much more quickly than DsRed (<1 hr) but is still a tetramer.

The spectral properties of mRFP, a monomerized variant of DsRed created by Campbell et al. (2002), make it substantially dimmer than DsRed (Table 21.4.2). Yet, the rate of folding for this protein is ten times faster, which effectively results in similar levels of brightness compared with wild-type DsRed in cells (Campbell et al., 2002).

It should be noted that while mRFP is generally less prone to aggregation than previous variants of DsRed, researchers in the Lippincott-Schwartz laboratory have encountered difficulties with the fusion of mRFP to integral membrane proteins. Often, bright red puncta can be observed in cells expressing these FFPs (Fig. 21.4.3C), while GFP-based FFPs do not form these puncta. The most likely explanation is that attachment of mRFP to a membrane protein reduces that protein's rotational diffusion, increases its effective concentration (in the two dimensions of the membrane), and results in a tendency to form aggregates. In general, when using a new FP to make an FFP, it is useful to compare the FFP distribution with the distribution of the native protein fused to an EGFP variant or the immunofluorescence distribution of the native protein alone.

Additional coral FPs include a far red–emitting FP, HcRed (Gurskaya et al., 2001), as well as a green-emitting FP (Karasawa et al., 2003), among others (Table 21.4.2). Some of these proteins are also obligate oligomers, and the investigator should be familiar with the properties of these FPs before deciding to use them.

Photoactivatable proteins and timer proteins: Not all FPs are restricted to a single color. As noted in the preceding section, wild-type DsRed undergoes a slow maturation involving a phase as a green-emitting FP. A variant, DsRed-E5, has a more rapid transition from green to red (within 5 hr of protein synthesis) and permits investigators to follow both the steady-state distribution and the relative age of an FFP (Terskikh et al., 2000). The ratio of more mature (red) proteins to immature (green) proteins can be used to obtain information about the lifetime of the protein and whether proteins of different ages have different distributions.

To mark discrete populations of proteins, three FP options are currently available—PAGFP (Patterson and Lippincott-Schwartz, 2002), Kaede (Ando et al., 2002), and KFP-1 (Chudakov et al., 2003), which are all photoactivatable FPs. Because a particular population of protein molecules tagged with one of these FPs can be photoactivated (i.e., the fluorescent properties of the FP tag can be “turned on”) at a discrete time, the new synthesis of fluorescent proteins during the time course of an experiment is eliminated as a potential problem. PAGFP is derived from GFP and is monomerized. Initially, it is excited by UV light (Patterson and Lippincott-Schwartz, 2002) and barely emits in the green range (505 to 530 nm). Upon brief stimulation with intense UV light (similar to a photobleaching experiment), however, it can be excited under the same conditions as EGFP and will fluoresce 100 times more brightly in the green range compared with the unstimulated protein. (Note that PAGFP is fluorescent in the blue range both before and after photoactivation.) UV photoactivation requires a 405-or 413-nm excitation laser or a mercury arc lamp with the proper filter set. For a more detailed description of working with this FP, see Patterson and Lippincott-Schwartz (2004). Once photoactivated, PAGFP can also be used for photobleaching applications, such as FRAP.

Kaede, a variant of DsRed, can be photoactivated with 350- to 415-nm excitation to increase its fluorescence intensity up to 1000-fold over background. Absorption shifts from 508 nm to 572 nm upon activation, and emission shifts from 518 nm to 582 nm (Ando et al., 2002). In contrast to PAGFP, Kaede undergoes a complete conversion in both its absorption and emission spectra, and this conversion readily permits the simultaneous spectral separation of activated and unactivated proteins. However, Kaede is an obligate tetramer and requires UV light for photoactivation. To avoid exposing cells to intense UV light, investigators can use the coral FP KFP-1, which can be excited with a less phototoxic, longer-wavelength laser (532 nm) to

increase fluorescence 30-fold over background (Chudakov et al., 2003). In addition, KFP-1 differs from the other two photoactivatable FPs in that it is inherently nonfluorescent in its unactivated form. However, like Kaede, KFP-1 is an obligate tetramer.

In addition to having pulse-labeling applications, photoactivatable proteins can be used to mark organelles or whole cells and may be useful for following cell lineages in development experiments.

Alternatives—As noted above, none of the FPs are small, and some proteins do not fold or are nonfunctional following the attachment of a bulky FP (Andresen et al., 2004). If this is the case, a few alternative options are available.

Fluorescent dyes: Prior to the cloning of GFP, investigators studying proteins or compartments in living cells often used organelle-specific dyes (e.g., MitoTracker; Invitrogen) or microinjected purified proteins conjugated to dyes such as fluorescein or rhodamine into cells. These methods are still used and can be quite powerful by themselves or in combination with FFPs. Dye labeled proteins offer certain advantages over natural FPs, including (1) tight control over the amount of fluorescently labeled protein in the cell; (2) the elimination of concerns about new fluorescent protein synthesis; (3) the ability to acutely introduce otherwise cytotoxic proteins into the cell; (4) the wide variety of available dyes, compared with the relatively small selection of FPs; and (5) the much smaller size of dye molecules, making them less likely to cause steric hindrance. The primary concerns with dye-labeled proteins are that (1) dyes may render a protein nonfunctional; (2) many readily undergo photobleaching, which can cause significant photodamage to cells (although some of the newer dyes, such as Invitrogen's Alexa dyes, are more resistant to photobleaching); (3) a microinjector is often required to deliver dye-labeled proteins to the cytoplasm; and (4) the lumena of many organelles cannot be accessed or labeled.

Short fluorophore-binding epitopes: Some of the smallest available options for fluorescently labeling proteins include fluorescent biarsenical-linked dyes (namely, FlAsH and ReAsH). The addition of a tetracysteine amino acid sequence (CCPGCC) to a flexible exposed domain of a protein of interest (typically the COOH-terminus) makes the protein competent to bind these fluorescein- (FlAsH) or rhodamine-derived (ReAsH) arsenicals (Griffin et al., 1998; Gaietta et al., 2002). These modified dyes are membrane-permeable and relatively small. Thus, it is possible to fluorescently label both cytoplasmic and lumenal proteins in cells without resorting to microinjection or other protein delivery methods. An important advantage is the comparatively small size of the tetracysteine tag and the bound dyes. Andresen et al. (2004) recently demonstrated that specific yeast tubulin isoforms were functional when one or two tetracysteine motifs were added, whereas complementation in mutant cells was not possible when three tetracysteine motifs or a single GFP was added.

Another useful property of these two dyes is that they can be added sequentially to differentially label newly synthesized proteins, resulting in a kind of biochemical photoactivation (Gaietta et al., 2002). Furthermore, ReAsH can photoconvert diaminobenzidine (DAB) to produce an electron-dense reaction product, permitting tetracysteine-tagged proteins to be imaged in the same cells by fluorescence, bright-field, and electron microscopy (Gaietta et al., 2002).

The tetracysteine-binding dyes do have at least one limitation associated with their use. Stroffekova et al. (2001) observed a significant fluorescence background even in FlAsH-treated cells not expressing a tetracysteine-tagged protein, as mammalian cells were found to contain proteins that naturally bind FlAsH. Those authors concluded that FlAsH labeling was best suited to proteins that are expressed at especially high levels (Stroffekova et al., 2001). However, proteins that concentrate in discrete cellular domains, such as gap junctions (Gaietta

et al., 2002), probably would not require substantial overexpression to be visualized over background. The biarsenical dyes are comparatively new and may have other limitations. Nonetheless, FlAsH and ReAsH represent viable alternatives to the more commonly used FPs and are worth considering.

Troubleshooting

See Table 21.4.3 for a guide to troubleshooting in FFP design and use.

Anticipated Results

The methods described in this unit should permit the investigator to generate a fluorescently labeled protein that is expressed at levels comparable to those for the native unlabeled protein, that targets to the correct compartment in the cell, and that exhibits behavior similar to that of the native protein (in terms of half-life, dynamics, and protein-protein interactions). The investigator may exploit the FFP in live cells, developing animals, or in solution.

Time Considerations

The time required for creation of an FFP will depend on the number of cloning steps required to generate the construct. If convenient restriction sites are present in the DNA sequence encoding the protein of interest and there are no concerns with targeting sequences, an FFP can be created in the time it takes to perform digestion, DNA purification, and ligation, followed by transformation and screening for the resulting DNA construct (1 to 3 days). More complex cloning strategies may require one or more PCR steps, the design of oligonucleotides, and more screening steps; in such cases, FFP creation can take up to 1 to 2 weeks. Characterization of the new construct with regard to proper localization will require the time necessary to transfect cells and perform immunocolocalization experiments (typically 4 hr to label cells and <1 hr to image them), and the time needed for functional assays will be application dependent.

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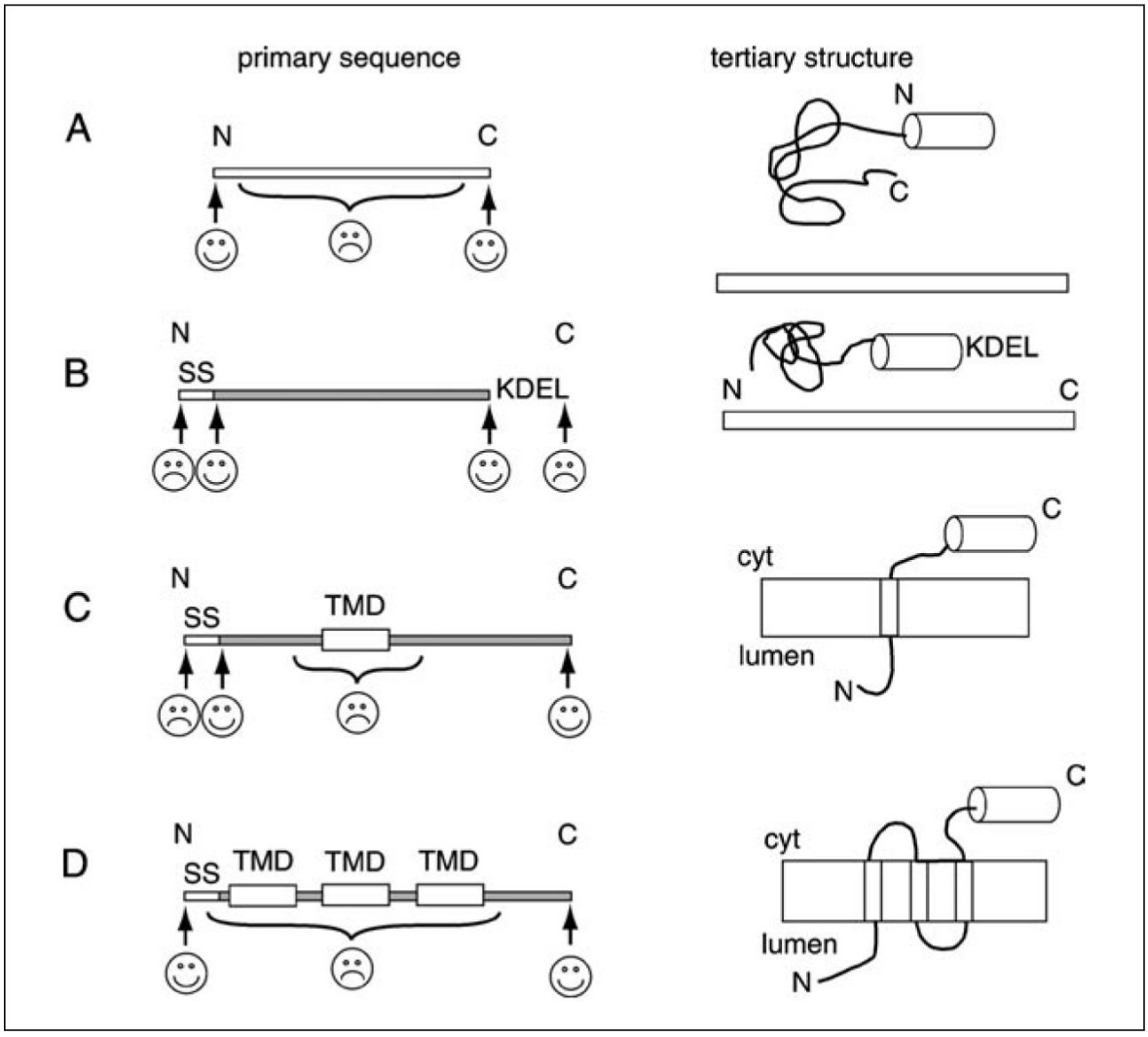

Figure 21.4.1.

Appropriate positioning of a fluorescent protein (FP) in a fluorescent fusion protein construct. Preferred sites of FP fusion in the primary sequence of the protein of interest are indicated by happy face icons, and domains to be avoided are indicated by sad face icons. Each tertiary structure shows the folding of the sample construct with the FP (represented as a cylinder) fused at an optimal site. (**A**). A hypothetical globular protein expressed in the cytoplasm can have the FP fused at either the NH_2– or the COOH-terminus. Typically, one end of the protein of interest will contain a functional domain that may be sterically hindered by an FP, and so it is useful to make both of the possible constructs. (**B**) A hypothetical lumenal protein contains an NH_2–terminal signal sequence (SS), a mature domain, and a COOH-retention sequence (KDEL). An FP placed immediately after the SS or immediately before the retention sequence is less likely to interfere with the functioning of either sequence. (**C**) A single membrane–spanning protein has the additional constraint that the FP cannot be placed within or near the transmembrane domain (TMD), as this will disrupt the domain and cause problems with membrane integration. (**D**) A membrane multispanning protein has the same constraint as the example in panel C, but in multiple locations. The loops between the transmembrane domains are also poor choices, because the exact spacing between transmembrane domains is often important for protein folding, and because these loops often contain functional domains. Abbreviation: cyt, cytoplasm.

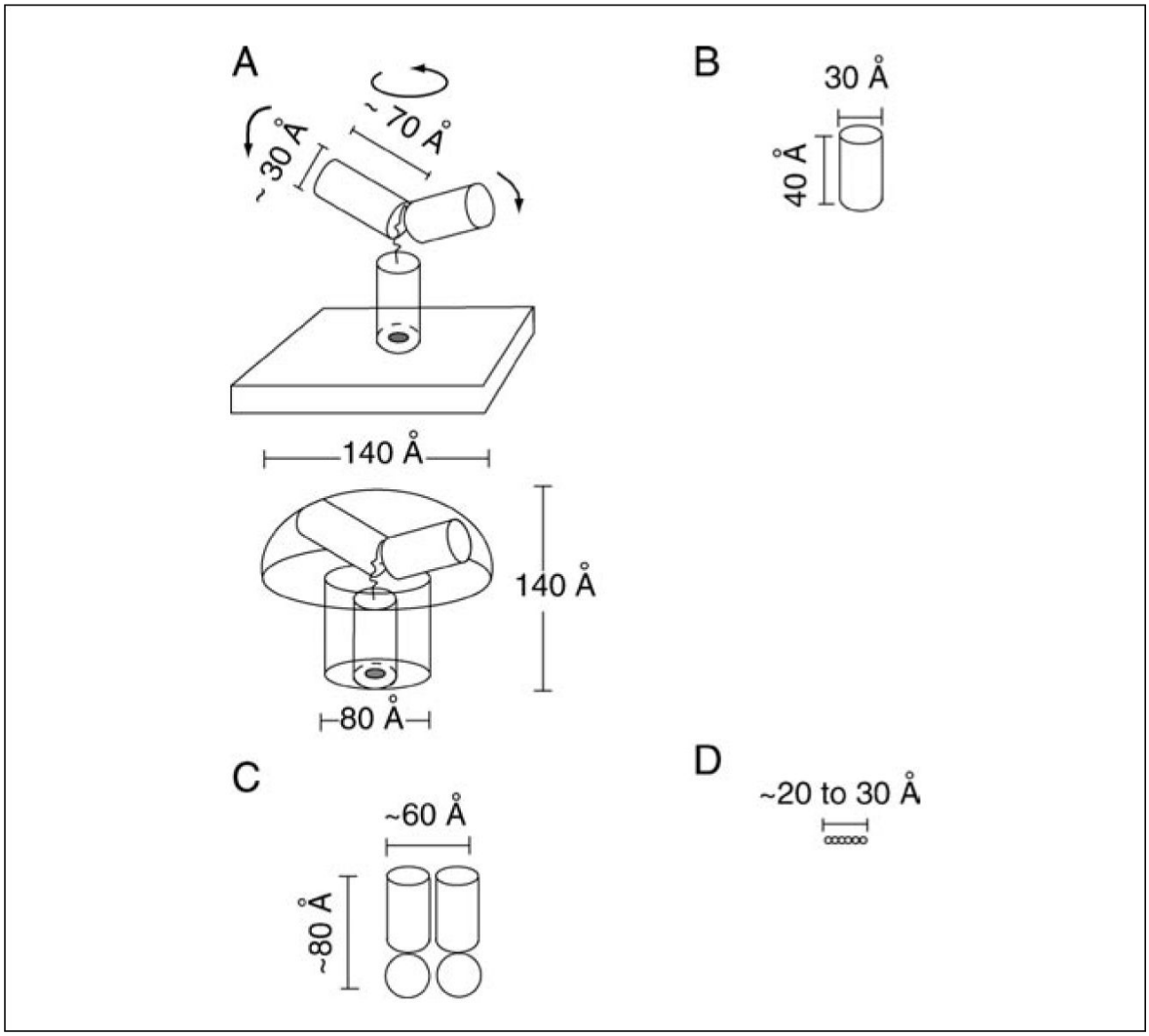

Figure 21.4.2.
Relative sizes of (**A**) immunoglobulin G (IgG; reference for comparison with panels B to D), (**B**) green fluorescent protein (GFP), (**C**) the *Discosoma* red fluorescent protein (DsRed) tetramer, and (**D**) biarsenical tetracysteine.

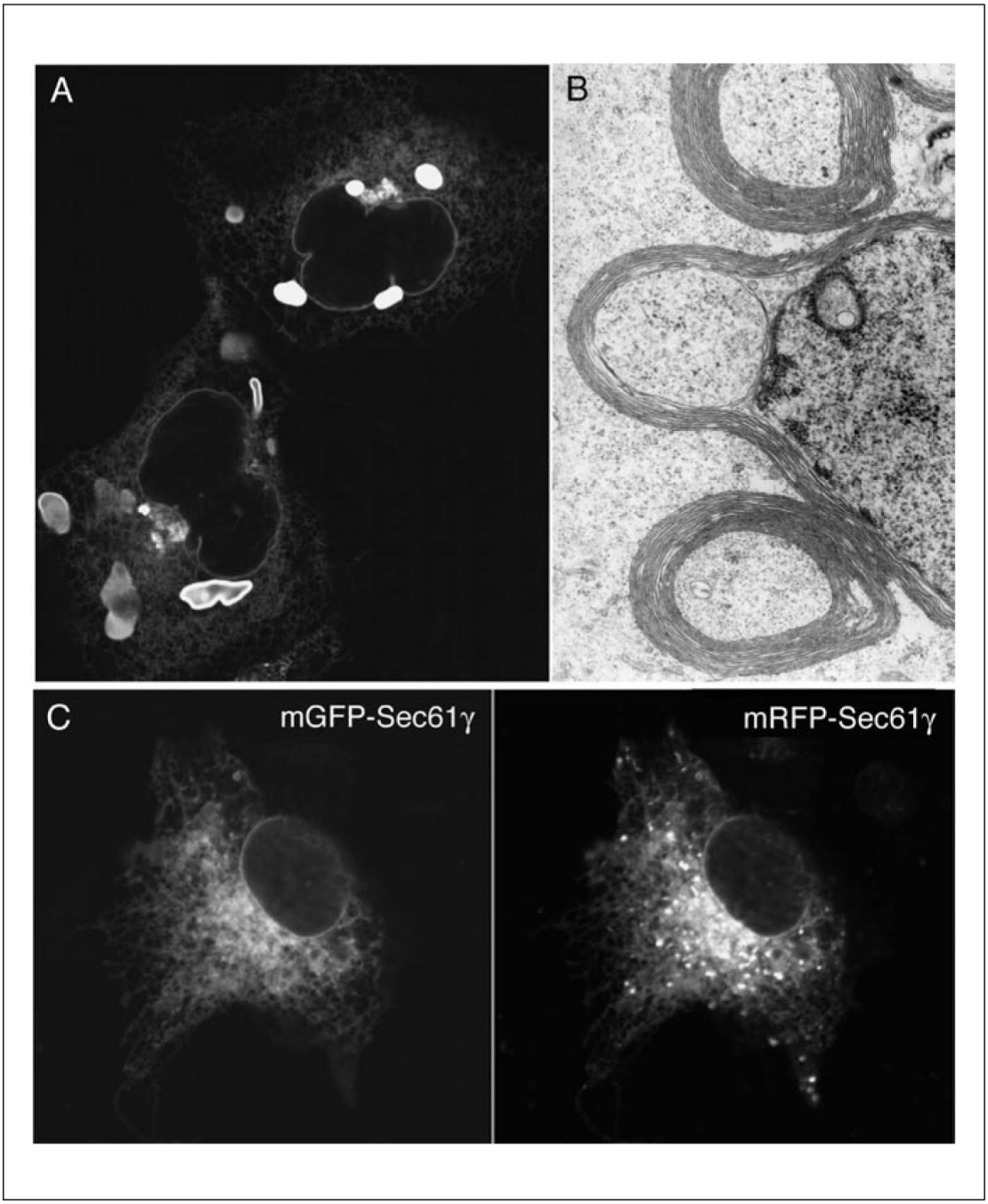

Figure 21.4.3.

The fusion of a fluorescent protein (FP) to a native protein may change the protein's normal localization pattern or may lead to the formation of aggregates or oligomers. (**A**) The fusion of nonmonomerized enhanced green fluorescent protein (EGFP) to a resident endoplasmic reticulum (ER) membrane protein induces the formation of an organized smooth ER structure. (**B**) The fusion of monomerized EGFP to the same protein does not grossly alter the structure of the ER. (**C**) A Cos-7 cell expressing two fluorescent fusion proteins (FFPs), one containing monomerized green fluorescent protein (mGFP; left-hand image) and the other containing monomerized red fluorescent protein (mRFP; right-hand image). The mGFP-containing FFP localizes to the ER network, and similarly, the mRFP-containing FFP colocalizes to the ER

membranes. The image yielded by the mRFP-tagged protein shows bright puncta, which are probably mRFP aggregates.

Table 21.4.1

Typical Protein Concentrations in Cells

Protein	Concentration[a,b] (μM)	Reference
Enzymes		
Ornithine decarboxylase	15 (rabbit liver)	Albe et al., 1990
Hexokinase	0.5 (rabbit muscle)	Albe et al., 1990
Aldolase	15 (rat liver); 809 (rabbit muscle)	Albe et al., 1990
Signaling molecules		
MAPKKK (Mos)	0.006 to 0.015	Huang and Ferrell, 1996
ras p21	0.25	Hand et al., 1987
MAPK phosphatase	0.024 to 0.6	Huang and Ferrell, 1996
MAPKinase (p42)	0.24 to 6	Huang and Ferrell, 1996
MAPKK (Mek1)	0.24 to 6	Huang and Ferrell, 1996
cAMP-dependent kinase	2	Francis and Corbin, 1994
Calmodulin	30	Manalan and Klee, 1984
Cytoskeleton		
Actin	95	Luby-Phelps, 2000
Vimentin	3	Luby-Phelps, 2000

[a] According to Niswender et al. (1995) and Patterson et al. (1997), a concentration of at least 200 nM enhanced green fluorescent protein (EGFP) is necessary to visualize a fluorescence increase over background autofluorescence in HeLa cells. Enhanced yellow fluorescent protein (EYFP) is 1.5× brighter than EGFP (Table 21.4.2), and so only 133 nM EYFP is necessary for a detectable fluorescence increase. See Table 21.4.2 for relative brightness data on other fluorophores.

[b] The volume of a BHK cell (3900 μm^3; Griffiths et al., 1984) was used to calculate concentration when only an absolute protein amount was provided in the literature.

Table 21.4.2

Properties of Commonly Used Fluorescent Proteins[a]

Fluorescent protein	Excitation maximum (nm)	Emission maximum (nm)	Relative brightness[b]	Reference
wtGFP	397	508	20,448 (0.6×)	Patterson and Lippincott-Schwartz, 2002
EGFP	490	509	33,000 (1×)	Rizzo et al., 2004
PAGFP (preactivation)	504	515	2691 (0.08×)	Patterson and Lippincott-Schwartz, 2002
PAGFP (postactivation)	400	517	13,746 (0.4×)	Patterson and Lippincott-Schwartz, 2002
EYFP	514	527	51,240 (1.5×)	Rizzo et al., 2004
Venus	515	528	52,554 (1.6×)	Nagai et al., 2002
Citrine	516	529	58,520 (1.8×)	Griesbeck et al., 2001
ECFP	433	476	10,730 (0.3×)	Rizzo et al., 2004
Cerulean Blue	433	475	26,660 (0.8×)	Rizzo et al., 2004
DsRed	558	583	45,030 (1.4×)	Campbell et al., 2002
T1	554	586	12,642 (0.4×)	Bevis and Glick, 2002
mRFP	584	607	11,000 (0.3×)	Campbell et al., 2002
Kaede (preactivation)	508	518	78,400 (2.4×)	Ando et al., 2002
Kaede (postactivation)	572	582	19,932 (0.6×)	Ando et al., 2002
HcRed	592	645	na	Gurskaya et al., 2001
KFP-1 (preactivation)	na	600	<123 (0.004×)	Chudakov et al., 2003
KFP-1 (postactivation)	580	600	4130 (0.1×)	Chudakov et al., 2003
mAzami-Green	492	505	33,858 (1×)	Karasawa et al., 2003

[a] Abbreviation: na, not available.

[b] Quantum yield multiplied by extinction coefficient. Number in parentheses is the brightness of the fluorescent protein relative to EGFP.

Table 21.4.3

Troubleshooting Guide for the Design and Use of Fluorescent Fusion Proteins[a]

Problem	Cause	Solution
FFP is not fluorescent	FFP's environment may suppress fluorescence (e.g., pH is too low)	Try an FP that is more tolerant of different environments
	FFP may not be folded	Place a short linker (2 to 10 amino acids) between the protein of interest and the FP; this may help to ensure proper folding of both the protein and the FP
	FFP is not synthesized or is highly unstable	Determine whether the FFP is expressed by performing immunoblot analysis (*UNIT 6.2*) or pulse-labeling/immunoprecipitation analysis (*UNITS 7.1* & *7.2*) of transfected cells[b]
FFP does not localize correctly	FP interferes with native protein's targeting sequence	Construct the FFP with the FP at the opposite end of the protein of interest
		Place a short linker (2 to 10 amino acids) between the protein of interest and the FP; this may help to ensure proper folding of both the protein and the FP
		Targeting machinery may be saturated; determine whether mistargeting is seen specifically in cells with higher expression of the FFP; if so, limit observations to dimmer cells[c]
	FP oligomerizes	Use a monomeric FP
FFP is nonfunctional	FP may sterically hinder folding or obstruct functional domains	Try constructing the FFP with the FP placed elsewhere in the protein of interest
		Place a short linker (2 to 10 amino acids) between the protein of interest and the FP; this may help to ensure proper folding of both the protein and the FP
		Try using a fluorescent biarsenical-linked dye (FlAsH or ReAsH) in place of the FP
		Try using a dye-labeled protein instead of an FFP

[a] Abbreviations: FFP, fluorescent fusion protein; FP, fluorescent protein.

[b] If the protein is expressed but unstable, the FP must be placed in a different position. Try fusing the FP gene near the other terminus of the cDNA encoding the protein of interest. Alternatively, the FP may prevent incorporation of the FFP into a complex, thereby reducing FFP stability. If this is suspected, consider FlAsH or other smaller fluorescent labels. If the FFP is not expressed, check the sequence of the construct again. If the sequence is correct and contains an appropriate Kozak sequence, then the flanking sequences of the FFP gene may be interfering with transcription or translation. If this is the case, the FFP coding sequence (with Kozak sequence) should be excised and inserted in the multicloning site of an appropriate expression vector.

[c] Overall expression can be reduced by stably transfecting cells and/or by placing the FFP construct in a vector with an inducible promoter and modulating FFP gene expression.