# Genetically encoded fluorescent tags

Kurt Thorn*
Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, CA 94158

**ABSTRACT** **Genetically encoded fluorescent tags are protein sequences that can be fused to a protein of interest to render it fluorescent. These tags have revolutionized cell biology by allowing nearly any protein to be imaged by light microscopy at submicrometer spatial resolution and subsecond time resolution in a live cell or organism. They can also be used to measure protein abundance in thousands to millions of cells using flow cytometry. Here I provide an introduction to the different genetic tags available, including both intrinsically fluorescent proteins and proteins that derive their fluorescence from binding of either endogenous or exogenous fluorophores. I discuss their optical and biological properties and guidelines for choosing appropriate tags for an experiment. Tools for tagging nucleic acid sequences and reporter molecules that detect the presence of different biomolecules are also briefly discussed.**

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## INTRODUCTION

Over the past two decades, genetically encoded fluorescent tags have revolutionized cell biology. These are protein sequences that fold and become fluorescent either by formation of a fluorophore from the protein sequence or by binding a small-molecule fluorophore. Intensive engineering of these proteins has led to a large variety of tags, with fluorescent colors spanning the visible spectrum, enabling multicolor imaging of nearly any set of proteins of interest by genetically fusing fluorescent tags to the proteins of interest. More recently, strategies have been developed for targeting specific RNA and DNA sequences, extending this to a larger class of biomolecules. Combined with modern light microscopy techniques (for an introduction, see Thorn, 2016), these tags provide a powerful way to interrogate biological processes. Although not discussed here, genetically encoded tags for electron microscopy are also available (Gaietta *et al.*, 2002; Shu *et al.*, 2011; Martell *et al.*, 2012; Kuipers *et al.*, 2015; Lam *et al.*, 2015).

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*Address correspondence to: Kurt Thorn (kurt.thorn@gmail.com).

Abbreviations used: CFP, cyan fluorescent protein; CRISPR, clustered regularly interspaced short palindromic repeats; DFHBI, 3,5-difluoro-4-hydroxybenzylidene imidazolinone; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; EYFP, enhanced yellow fluorescent protein; GFP, green fluorescent protein; mEGFP, monomeric EGFP; QY, quantum yield; TALE, transcription activator-like effector; TMP, trimethoprim; UV, ultraviolet; YFP, yellow fluorescent protein.



## TYPES OF FLUORESCENT TAGS

Fluorescent proteins can broadly be divided into three classes, based on the origin of the fluorophore, the chemical moiety that absorbs excitation light and then reemits emission light: Intrinsically fluorescent proteins, which become fluorescent after folding without addition of a fluorophore; extrinsically fluorescent proteins that bind an endogenous biomolecule as a fluorophore; and extrinsically fluorescent proteins that require addition of an exogenous (synthetic) ligand as a fluorophore. In many ways, intrinsically fluorescent proteins are the easiest to use: provided they fold in the environment in which they are expressed, they will fluoresce. However, the absence of an external fluorophore limits flexibility; the only way to change the fluorescence properties of the tag is by protein engineering. Proteins that bind an endogenous molecule as a fluorophore are as easy to use as intrinsically fluorescent proteins, provided that the fluorophore is present in the cell of interest. The proteins that have been developed in this class typically use widely distributed molecules as fluorophores, but sometimes the molecule must be supplemented exogenously, or enzymes to produce the fluorophore must be added to the cell. Finally, tags that bind an exogenous ligand typically use synthetic molecules. In many of these cases, the fluorophore is separate from the part of the molecule that binds the tag, allowing the fluorescence properties of the tag to be varied by changing the fluorophore.

## INTRINSICALLY FLUORESCENT PROTEINS

Green fluorescent protein (GFP) is the canonical example of an intrinsically fluorescent protein (for an introduction to and a history of GFP, see Chalfie, 2009; Tsien, 2009). It is a 238–amino acid protein that folds into an 11-stranded β-barrel (Figure 1). All known intrinsically

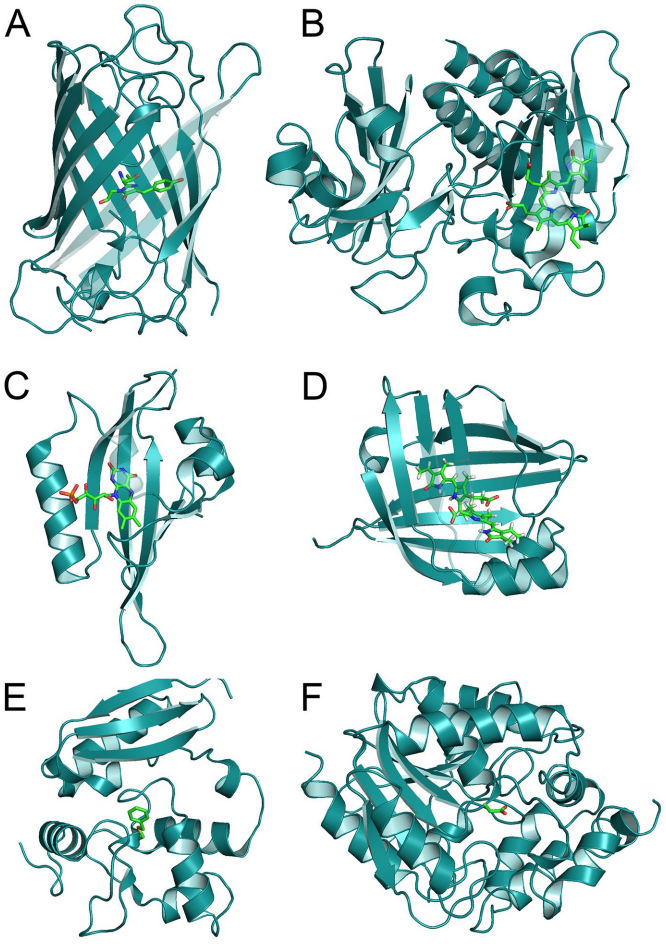


FIGURE 1: Structures of representative fluorescent tags. In all cases, the protein backbone is shown as a cartoon, and the fluorophore is drawn as a stick model (green, carbon; blue, nitrogen; red, oxygen; orange, phosphorus). In some cases, parts of the protein cartoon have been made transparent to better show the fluorophore. (A) Green fluorescent protein, an intrinsically fluorescent protein (Protein Data Bank [PDB] ID 1EMA; Ormö *et al.*, 1996). (B) IFP2.0, an extrinsically fluorescent protein that binds biliverdin as the fluorophore (PDB ID 4CQH; Yu *et al.*, 2014). (C) iLOV, an extrinsically fluorescent protein that binds flavin mononucleotide as the fluorophore (PDB ID 4EES; Christie *et al.*, 2012). (D) UnaG, an extrinsically fluorescent protein that binds bilirubin as the chromophore (PDB ID 4I3B; Kumagai *et al.*, 2013). (E) SNAP tag, an extrinsically fluorescent protein that labels itself with benzyl derivatives. The benzyl group where the fluorophore would be attached is shown as a stick model (PDB ID 3L00; Mollwitz *et al.*, 2012). (F) Halo tag, an extrinsically fluorescent protein that labels itself with alkyl halide derivatives. The aspartate residue where the label would be attached is shown as a stick model (PDB ID 1BN6; Newman *et al.*, 1999).

fluorescent proteins share the same fold and have similar mechanisms of fluorophore formation. After folding, a fluorophore is formed autocatalytically from three amino acids of the β-barrel (Tsien, 1998). Although the fluorophore of intrinsically fluorescent proteins is formed from the protein backbone, its formation requires oxygen, and so these proteins will not become fluorescent in anaerobic environments. In addition, the rates of protein folding and fluorophore formation vary depending on the protein and can be as short as a few minutes (Iizuka *et al.*, 2011) or as long as a few days (Baird *et al.*, 2000).

Originally isolated from the jellyfish *Aequorea victoria*, GFP is a member of a large family of fluorescent proteins sharing the β-barrel fold (Labas *et al.*, 2002; Alieva *et al.*, 2008). Unusually for this family, GFP is nearly monomeric, allowing it to be fused to a wide variety of proteins without changing their aggregation status, which contributed to its rapid and wide adoption. GFP was first engineered to improve its brightness (leading to enhanced GFP [EGFP], which is still widely used), and mutations to its chromophore and surrounding sequence shifted its spectrum to blue, cyan, and yellow variants (Tsien, 1998, 2009). Although (E)GFP and related molecules dimerize only weakly ($K_d \approx 110$ μM), this dimerization causes problems in some assays. Mutation of residues at the dimer interface reduces the $K_d$ by nearly 1000-fold, resulting in monomeric EGFP (mEGFP), which shows negligible dimerization (Zacharias *et al.*, 2002). GFP stubbornly resisted engineering to a red fluorescent protein, and existing red fluorescent proteins result from substantial engineering of tetrameric proteins to produce bright-red monomeric fluorescent proteins (Shaner *et al.*, 2004; Merzlyak *et al.*, 2007; Shcherbo *et al.*, 2009). These proteins share the same β-barrel fold as GFP and the same autocatalytic mechanism of fluorophore formation, although the fluorophores differ in structure, producing the different spectral properties of these proteins.

Good intrinsically fluorescent proteins, derived from a range of sources, are available in colors from blue (400-nm excitation/450-nm emission) to far red (600-nm excitation/630-nm emission). However, despite many attempts, no bright, intrinsically fluorescent near-infrared fluorescent protein (one with a peak emission >700 nm) has been developed; the best near-infrared fluorescent proteins available use extrinsic fluorophores. Protein engineering has been used to optimize many other properties of fluorescent proteins, including folding time (Fisher and DeLisa, 2008; Iizuka *et al.*, 2011), protein stability (Pédelacq *et al.*, 2006), and photostability (Shaner *et al.*, 2008; Ren *et al.*, 2016). The result is a large range of fluorescent proteins from which to choose, with properties optimized for specific applications. Table 1 lists a selection of widely used fluorescent proteins, but many more are available. More comprehensive lists can be found (www.fpvis.org; Chudakov *et al.*, 2010; Cranfill *et al.*, 2016; Rodriguez *et al.*, 2016a).

## EXTRINSICALLY FLUORESCENT PROTEINS WITH ENDOGENOUS LIGANDS

More recently, as the limits of intrinsically fluorescent protein development have become to be realized, attention has turned to the development of fluorescent proteins that use endogenous biomolecules as fluorophores. Specifically, substantial effort has focused on developing variants of bacterial phytochromes and phycobiliproteins as near-infrared fluorescent proteins (Figure 1). These proteins naturally bind the heme degradation product biliverdin or closely related molecules and fluoresce weakly in the infrared. Engineering of these proteins has led to much brighter fluorescence at wavelengths commonly used for imaging and to proteins that are less perturbative in fusions. However, development of these proteins is less advanced than that of intrinsically fluorescent proteins, and many of the brightest proteins are dimers (Shcherbakova and Verkhusha, 2013; Rodriguez *et al.*, 2016b). In some cases, functional monomers can be produced from these proteins by fusing two copies of the sequence to form a tandem dimer (Rodriguez *et al.*, 2016b). In addition, although these proteins use an endogenous molecule, biliverdin, as a fluorophore, fluorescence can often be increased by supplementing cells with additional biliverdin or cell-permeant analogues (Rodriguez *et al.*, 2016b) or by coexpressing heme oxygenase to increase the intracellular concentration of biliverdin (Yu *et al.*, 2014).

Development of extrinsically fluorescent proteins has not been limited to infrared fluorescent proteins. There is also a green

| Name | $\lambda_{ex}$ (nm) | $\lambda_{em}$ (nm) | $E$ ($mM^{-1}$ $cm^{-1}$) | QY | $E \times QY$ | Type | $pK_a$ | Bleach time (s) | Reference |
|---|---|---|---|---|---|---|---|---|---|
| Intrinsically fluorescent proteins | | | | | | | | | |
| mTagBFP2 | 399 | 454 | 51 | 0.64 | 32.4 | m | 2.7 | 53 | Subach *et al.* (2011) |
| mTurquoise2 | 434 | 474 | 30 | 0.93 | 27.9 | m | 3.1 | 90 | Goedhart *et al.* (2012) |
| mCerulean3 | 433 | 475 | 40 | 0.80 | 32 | m | 4.7 | | Markwardt *et al.* (2011) |
| EGFP | 488 | 507 | 56 | 0.60 | 33.6 | m | 6.0 | 174 | Yang *et al.* (1996) |
| mWasabi | 493 | 509 | 70 | 0.80 | 56 | m | 6.0 | 93 | Ai *et al.* (2008) |
| Superfolder GFP | 485 | 510 | 83 | 0.65 | 54.1 | m | | | Pédelacq *et al.* (2006) |
| mNeonGreen | 506 | 517 | 116 | 0.80 | 92.8 | m | 5.7 | 158 | Shaner *et al.* (2013) |
| mClover3 | 506 | 518 | 109 | 0.78 | 85 | m | 6.5 | 80 | Bajar *et al.* (2016b) |
| Venus | 515 | 528 | 92 | 0.57 | 52.5 | m | 6.0 | 15 | Nagai *et al.* (2002) |
| Citrine | 516 | 529 | 77 | 0.76 | 58.5 | m | 5.7 | 49 | Griesbeck *et al.* (2001) |
| mKOκ | 551 | 563 | 105 | 0.61 | 64 | m | 4.2 | | Tsutsui *et al.* (2008) |
| tdTomato | 554 | 581 | 138 | 0.69 | 95.2 | td | 4.7 | 98 | Shaner *et al.* (2004) |
| TagRFP-T | 555 | 584 | 81 | 0.41 | 33.2 | m | 4.6 | 337 | Shaner *et al.* (2008) |
| mRuby3 | 558 | 592 | 128 | 0.45 | 57.6 | m | 4.8 | 349 | Bajar *et al.* (2016b) |
| mScarlet | 569 | 594 | 100 | 0.70 | 70.0 | m | 5.3 | 277 | Bindels *et al.* (2017) |
| FusionRed | 580 | 608 | 95 | 0.19 | 18.1 | m | 4.6 | 150 | Shemiakina *et al.* (2012) |
| mCherry | 587 | 610 | 72 | 0.22 | 15.8 | m | 4.5 | 96 | Shaner *et al.* (2004) |
| mStable | 597 | 633 | 45 | 0.17 | 7.6 | m | | 1002 | Ren *et al.* (2016) |
| mKate2 | 588 | 633 | 63 | 0.40 | 25 | m | 5.4 | 84 | Shcherbo *et al.* (2009) |
| mMaroon1 | 609 | 657 | 80 | 0.11 | 8.8 | m | 6.2 | 178 | Bajar *et al.* (2016a) |
| mCardinal | 604 | 659 | 87 | 0.19 | 16.5 | m | 5.3 | 730 | Chu *et al.* (2014) |
| T-Sapphire | 399 | 511 | 44 | 0.60 | 26.4 | m | 4.9 | 25 | Zapata-Hommer and Griesbeck (2003) |
| mCyRFP1 | 528 | 594 | 27 | 0.65 | 18 | m | 5.6 | 45 | Laviv *et al.* (2016) |
| LSSmOrange | 437 | 572 | 52 | 0.45 | 23.4 | m | 5.7 | | Shcherbakova *et al.* (2012) |
| mBeRFP | 446 | 611 | 65 | 0.27 | 17.6 | m | 5.6 | | Yang *et al.* (2013) |
| Extrinsically fluorescent proteins with endogenous ligands | | | | | | | | | |
| CreiLOV | 450 | 495 | 12 | 0.51 | 6.4 | m | 3.0 | | Mukherjee *et al.* (2015) |
| UnaG | 498 | 527 | 77 | 0.51 | 39.4 | m | 4.0 | | Kumagai *et al.* (2013) |
| miRFP670 | 642 | 670 | 71 | 0.12 | 8.5 | m | 4.5 | 155 | Shcherbakova *et al.* (2016) |
| TDsmURFP | 642 | 670 | 170 | 0.18 | 30.6 | td | | 190 | Rodriguez *et al.* (2016b) |
| iRFP670 | 643 | 670 | 114 | 0.11 | 12.5 | d | 4.0 | | Shcherbakova and Verkhusha (2013) |
| mIFP | 683 | 704 | 82 | 0.08 | 6.6 | m | 3.5 | | Yu *et al.* (2015) |
| iFP2.0 | 690 | 711 | 86 | 0.08 | 6.9 | m | | | Yu *et al.* (2014) |
| iRFP720 | 702 | 720 | 96 | 0.06 | 5.8 | d | 4.5 | | Shcherbakova and Verkhusha (2013) |
| Extrinsically fluorescent proteins with exogenous ligands | | | | | | | | | |
| FlAsH/peptide[a] | 508 | 528 | 70 | 0.85 | 59.5 | m | | | Martin *et al.* (2005) |
| ReAsH/peptide[a] | 593 | 608 | 69 | 0.48 | 33.1 | m | | | Martin *et al.* (2005) |
| TO1/scFv[b] | 509 | 530 | 60 | 0.47 | 28.2 | m | | | Szent-Gyorgyi *et al.* (2008) |
| MG/scFv[c] | 635 | 656 | 105 | 0.25 | 26.3 | m | | | Szent-Gyorgyi *et al.* (2008) |

**TABLE 1:** Selected fluorescent molecules.

(Continues)

| Name | $\lambda_{ex}$ (nm) | $\lambda_{em}$ (nm) | $E$ ($mM^{-1}$ $cm^{-1}$) | QY | $E \times QY$ | Type | $pK_a$ | Bleach time (s) | Reference |
|---|---|---|---|---|---|---|---|---|---|
| Commonly used small-molecule fluorophores (e.g., for self-labeling tags) | | | | | | | | | |
| Atto 488 | 500 | 520 | 90 | 0.80 | 72.0 | | | | www.atto-tec.com |
| $JF_{549}$ | 549 | 571 | 101 | 0.88 | 88.9 | | | | Grimm *et al.* (2015) |
| Alexa 568 | 578 | 603 | 88 | 0.69 | 60.7 | | | | www.thermofisher.com |
| $JF_{646}$ | 646 | 664 | 152 | 0.54 | 82.1 | | | | Grimm *et al.* (2015) |
| Alexa 647 | 650 | 668 | 270 | 0.33 | 89.1 | | | | www.thermofisher.com |

The proteins were selected to include the best available fluorescent proteins in common wavelength ranges, as well as examples of fluorescent proteins from unique classes or with unique properties. The small-molecule dyes shown are a very small subset of the large number of available dyes and may not be the best for any particular application. $\lambda_{ex}$ and $\lambda_{em}$ are the excitation and emission maxima, respectively. $E$ is the extinction coefficient, QY is the quantum yield, and $E \times QY$ is their product. $pK_a$ is the pH at which fluorescence is 50% quenched. Type: m, monomer; td, tandem dimer; and d, dimer. Bleach time is the time to bleach to half of the initial intensity at an initial emission rate of 1000 photons/s.

[a]FlAsH and ReAsH quantum yields and extinction coefficients are for the molecules bound to the peptide FLNCCPGCCMEP.

[b]TO1-2p/HL1.0.1-TO1 complex.

[c]MG-2p/H6-MG complex.

**TABLE 1:** Selected fluorescent molecules. Continued

fluorescent protein, UnaG, which uses another heme breakdown product, bilirubin, as the fluorophore (Kumagai *et al.*, 2013), as well as green fluorescent proteins that use flavins as the fluorophore (Drepper *et al.*, 2007; Chapman *et al.*, 2008; Buckley *et al.*, 2015). In practice, proteins that bind an endogenous fluorophore are used very similarly to intrinsically fluorescent proteins, although fluorophore concentration in the cell may need to be considered, as mentioned earlier. Although less bright, the green fluorescent flavin-binding proteins do have some advantages over EGFP: they are smaller than EGFP and do not require oxygen to become fluorescent. This last property makes them particularly valuable for imaging anaerobic organisms, where intrinsically fluorescent proteins do not mature. A selection of extrinsically fluorescent proteins is given in Table 1.

## EXTRINSICALLY FLUORESCENT PROTEINS WITH EXOGENOUS LIGANDS

Analogous to the proteins that bind endogenous fluorophores, proteins that bind exogenous fluorophores have also been developed. The best characterized of these are fluorogen-activating proteins, single-chain antibodies that bind a nonfluorescent molecule and stabilize it in a fluorescent state (Szent-Gyorgyi *et al.*, 2008; Bruchez, 2015). These are commercially available with both cell-permeant and cell-impermeant ligands, enabling discrimination of intracellular fusions from extracellular fusions. A related labeling scheme is that of FlAsH/ReAsh, in which a six–amino acid tetracysteine motif recognizes arsenic-containing dyes (Griffin *et al.*, 1998; Gaietta *et al.*, 2002). By themselves, the dyes are not fluorescent, but they become fluorescent when bound to the tetracysteine tag. Nonspecific binding to cysteines can lead to background fluorescence and is suppressed by washing with sulfhydryl-containing compounds. Although not widely used, this labeling scheme is noteworthy because the tag is very small. Spectral properties for these tags are given in Table 1.

An alternative way to fluorescently label a protein of interest is by covalently coupling a dye molecule to it. Although this has long been done in vitro using amine- or sulfhydryl-reactive dyes, more recently, self-labeling tag sequences have been used for this. These tags covalently react with a small-molecule substrate containing a fluorophore (Table 2). The most widely used tags are the SNAP(f), CLIP(f), and Halo tags (Keppler *et al.*, 2003; Gautier *et al.*, 2008; Los *et al.*, 2008; Sun *et al.*, 2011). The SNAP and CLIP tags are variants of $O^6$-alkylguanine-DNA alkyltransferase that react with benzylguanine and benzylcytosine derivatives, respectively (Figure 1). The Halo tag is derived from haloalkane dehalogenase and reacts with alkylhalides. A similar but less widely used tag is the TMP tag, which uses an engineered *Escherichia coli* dihydrofolate reductase to react with trimethoprim-fused fluorophores (Miller *et al.*, 2005; Chen *et al.*, 2012). In these systems, the reactive group that covalently binds to the tag is independent of the attached fluorophore, allowing a wide variety of fluorophores (and other molecules, such as affinity tags) to be attached. This chemical versatility allows changing the label on the protein by simply changing the substrate and enables experiments that would be difficult to carry out with other tags, such as two-color pulse-chase labeling by first incubating with one substrate and then by a second, or distinguishing intracellular from extracellular protein by labeling with cell-permeant and cell-impermeant substrates. The major drawback to these proteins is the added complexity of using an external substrate that is itself fluorescent and may require washing to reduce background, although there is a version of the TMP tag that reacts with nonfluorescent substrates to produce fluorescent adducts (Jing and Cornish, 2013). In addition, newly synthesized protein is fluorescently labeled only if substrate is available, which makes these methods less useful for long time-lapse imaging experiments.

## CHOOSING TAGS

The most common application of these tags is to follow the abundance, localization, and/or movement of a protein of interest using microscopy or flow cytometry. Most of these applications will be well served by intrinsically fluorescent proteins, possibly in combination with an infrared extrinsically fluorescent protein. When designing an experiment from scratch with the choice of any fluorescent protein(s) as tags, there are a number of parameters to consider: the spectral properties (color) of the fluorescent protein, its brightness and photostability, whether it affects the behavior of the protein to which it is fused, and how fast it matures.

## MULTICOLOR IMAGING

For many experiments, the first consideration will be what color (spectral properties) of fluorescent tag to use. The spectral properties of a fluorophore are given by its excitation and emission spectra. The excitation spectrum describes the wavelengths of light that,

| Tag | Description | Reference |
|---|---|---|
| Protein tags | | |
| Intrinsic and extrinsic fluorescent proteins | See Table 1 | |
| SNAP tag | 20 kDa; covalently labeled by reaction with benzylguanine derivatives | Keppler *et al*. (2003) |
| $SNAP_f$ tag | 20 kDa; covalently labeled by reaction with benzylguanine derivatives; faster labeling than SNAP tag | Sun *et al*. (2011) |
| CLIP tag | 20 kDa; covalently labeled by reaction with benzylcytosine derivatives | Gautier *et al*. (2008) |
| $CLIP_f$ tag | 20 kDa; covalently labeled by reaction with benzylcytosine derivatives; faster labeling than CLIP tag | Sun *et al*. (2011) |
| Halo tag | 33 kDa; covalently labeled by reaction with haloalkane derivatives | Los *et al*. (2008) |
| TMP tag | Engineered *E. coli* dihydrofolate reductase that binds trimethoprim-fluorophore conjugates | Miller *et al*. (2005), Chen *et al*. (2012), Jing and Cornish (2013) |
| SunTag | 73-kDa tag that recruits up to 24 GFPs | Tanenbaum *et al*. (2014) |
| GFP1-10/GFP11 and sfCherry1-10/sfCherry11 | 19–amino acid peptide from GFP that recruits remaining 222–amino acid GFP sequence; sfCherry is red equivalent; small size enables multimerization or CRISPR knock-in | Kamiyama *et al*. (2016), Leonetti *et al*. (2016) |
| RNA tags | | |
| F30-Broccoli | Green fluorescent RNA aptamer; binds exogenous fluorophore | Filonov *et al*. (2015) |
| Mango | Orange or red fluorescent RNA aptamer, depending on exogenous fluorophore | Dolgosheina *et al*. (2014) |
| DNB aptamer | Dinitrobenzyl-binding aptamer, enabling light-up labeling of RNA molecules | Arora *et al*. (2015) |
| JX1 | Benzylguanine-binding RNA aptamer, allowing use of SNAP-tag reagents for RNA labeling | Xu *et al*. (2016) |

Modular tags for protein and RNA sequences that are discussed in the text are listed here. For more information, see the text.

**TABLE 2:** Other genetically encoded tagging strategies.

when absorbed, will result in the fluorophore reemitting light; the spectrum of that emitted light is the emission spectrum. These are often reduced to numbers describing the peak excitation and emission wavelengths, but the spectra are often broad, and this simplification can obscure important information. To image two fluorescent tags in different channels requires that the excitation and emission spectra of one tag be sufficiently separate from those of the other tag (typically 60–100 nm) so that filters can be chosen that selectively detect each protein.

In many cases, the choice of colors is dictated by the instrumentation to which one has access and the fluorescent proteins it is designed to detect. For example, nearly all of the microscopes in the imaging center I direct can image blue, green, red, and infrared fluorescent proteins, but only a few microscopes are equipped with filters for cyan and yellow fluorescent proteins (CFP and YFP, respectively). I advise users of our center to avoid CFP and YFP if possible to maximize their imaging options. This is particularly true for instruments using laser illumination such as confocal, total internal reflection fluorescence, and light-sheet microscopes and flow cytometers, for which changing excitation wavelengths is expensive and difficult.

In general, I recommend starting with green and red fluorescent proteins, as these tend to be the brightest and best studied and have been found to work well for many applications. Somewhat surprisingly, EGFP still performs well in many systems, although newer proteins such as mClover3 and mNeonGreen outperform it in mammalian cells (Shaner *et al*., 2013; Bajar *et al*., 2016b). For red fluorescent proteins, mCherry was for many years the protein of choice, but it is now being supplanted by brighter and more photostable proteins. It appears likely that mScarlet (Bindels *et al*., 2017) will be the new red fluorescent protein of choice, but other proteins, such as mRuby3, TagRFP-T, and mKate2, may be worth considering (Shaner *et al*., 2008; Shcherbo *et al*., 2009; Bajar *et al*., 2016b). If additional colors are needed, mTagBFP2 (Subach *et al*., 2011) can be used with these with minimal cross-talk, although it is less bright than EGFP and phototoxicity is a concern with the near-ultraviolet (UV) excitation required. For a fourth color, a near-infrared fluorescent protein can be used, although these require a ligand. tdsmURFP is the brightest near-infrared fluorescent protein, although it requires supplementation with a biliverdin methyl ester to achieve maximum brightness (Rodriguez *et al*., 2016b). The next brightest monomeric option is mIRFP670 (Shcherbakova *et al*., 2016). However, both of these proteins are very new and have not been studied extensively. An alternative option is to use a self-labeling tag like Halo tag and an infrared dye; these dyes, such as Cy5 or Alexa 647, are substantially

brighter than the infrared fluorescent proteins. Four-color imaging with this combination is relatively straightforward, as both the fluorescent proteins and relevant filter sets are readily available.

CFP, YFP, and a red fluorescent protein can be used for three-color imaging (Livet *et al.*, 2007), and an alternate system for four-color imaging has recently been demonstrated with mTurquoise2, Clover, mKO2, and mMaroon1 (Bajar *et al.*, 2016a). In principle, both of these combinations should be extensible with the incorporation of a blue and infrared fluorescent protein to five and six colors. However, these combinations are less well tested, and filter sets for them are not widely available.

Other possibilities for imaging more than four fluorescent proteins at once include the use of long–Stokes shift proteins, which have a large separation between their excitation and emission wavelengths, enabling multiplexing with short–Stokes shift proteins. For example, T-Sapphire is a UV-excited GFP variant that can be multiplexed with the mWasabi green fluorescent protein, which is not UV excited (Ai *et al.*, 2008). This pair can be further combined with mTagBFP2, allowing three proteins to be imaged in the spectral space previously used for two. The near-infrared proteins iRFP670 and iRFP720 are sufficiently spectrally separate to allow two-color imaging (Shcherbakova and Verkhusha, 2013), suggesting that iRFP720 could be multiplexed with other near-infrared fluorescent proteins to access an additional channel. However, iRFP720 is dimeric, and filter sets for this protein are not common, complicating use of this option. Another option for multiplexing larger numbers of colors is to acquire fluorescence at many wavelengths and use computational tools to separate overlapping fluorophore spectra (Zimmermann, 2005; Cutrale *et al.*, 2017), although this requires specialized hardware and software.

## BRIGHTNESS

Another major consideration when choosing a protein tag is the brightness of the tag. Typically, this is measured by the product of the extinction coefficient (how effectively the fluorophore absorbs light) and the quantum yield (what fraction of absorbed photons produce a fluorescence). In general, the larger this product is, the brighter is the tag. However, these parameters are typically measured on pure protein in buffer and so may not be representative of what is observed in a cell. Protein tags must first fold; intrinsically fluorescent proteins then must form their fluorophore (mature), and extrinsically fluorescent proteins must bind their fluorophore. The folding and maturation rates of intrinsically fluorescent proteins can vary from minutes to hours and are not always well characterized. If fused to a short-lived protein, an otherwise bright protein may be quite dim, as it does not have time to mature and become fluorescent before being degraded. Similarly, the folding efficiency of the protein may vary depending on the target to which it is fused and the cell type in which it is expressed.

For extrinsically fluorescent proteins, the brightness of the tag will depend on the abundance of the extrinsic chromophore. In the case of biliverdin-binding proteins, this may require either supplementation with biliverdin or overexpression of an enzyme to increase the biliverdin concentration. Biliverdin levels vary among cells, and so the need for supplementation can be expected to vary as well. In the case of cells with cell walls (fungi, plants, bacteria) or in whole organisms, permeability of the extrinsic fluorophore can be expected to be an issue as well.

The environment of the protein (pH, salt concentration, etc.) can also affect tag brightness. The fluorescence of many proteins is quenched at low pH, which can cause problems when imaging in acidic environments. Many early EGFP and EYFP variants suffered from this problem, which was also exploited to produce pH sensors (Miesenböck *et al.*, 1998). EYFP also suffers from quenching by moderate concentrations of chloride ion, which was removed in the Citrine variant (Griesbeck *et al.*, 2001). The pH quenching is characterized for most new proteins, with the $pK_a$ (pH at which fluorescence is reduced by half) reported, but quenching by other ions is typically not characterized.

Because of the many factors that can affect tag brightness, it can be difficult to extrapolate from the measured brightness of pure protein to how a tag will perform in a particular organism. The brightness of pure fluorescent proteins is poorly correlated with brightness of protein fusions in both *Saccharomyces cerevisiae* and *Caenorhabditis elegans* (Lee *et al.*, 2013; Heppert *et al.*, 2016). However, few data are available for many tags and organisms, and so, if tag performance is critical for an experiment, it might be advisable to try multiple tags. Finally, when maximizing brightness is critical, as in single-molecule experiments, one should consider using self-labeling tags with exogenous fluorophores. Synthetic dyes are brighter and more photostable than fluorescent proteins and typically outperform them in these assays (Grimm *et al.*, 2015). For very bright tagging of single proteins, systems have also been developed for introducing multiple copies of small tags that then each recruit one or more fluorescent molecules (Tanenbaum *et al.*, 2014; Kamiyama *et al.*, 2016). If only a few copies of the tag are introduced, the tag size is small, enabling easy introduction of the tag using clustered regularly interspaced short palindromic repeats (CRISPR) technology (Leonetti *et al.*, 2016).

## PHOTOSTABILITY

A related issue is tag photostability. When excited, all fluorescent molecules can undergo side reactions leading to destruction of the fluorophore. This leads to loss of fluorescence over time and is referred to as photobleaching. How rapidly photobleaching occurs depends nonlinearly on the intensity of the excitation light and the length of time the fluorophore is illuminated (Cranfill *et al.*, 2016). Different fluorophores vary considerably in how rapidly they are photobleached, but under typical microscopy conditions, many fluorescent proteins are bleached by half after a few hundred seconds of continuous illumination. How much of a problem this is depends on the experiment. For short measurements, photobleaching poses little concern, but for time-lapse imaging, in which many images are acquired in a short period of time, it may be a critical factor. In these kinds of experiments, it may be better to use a more photostable protein that is less bright. Some proteins have been specifically optimized for photostability and may be worth considering for these experiments (Shaner *et al.*, 2008; Ren *et al.*, 2016).

Fluorescent molecules also produce reactive oxygen species when excited. These reactive oxygen species can contribute to phototoxicity, by which light exposure is toxic to cells. In general, shorter-wavelength light is more phototoxic than longer-wavelength light. Expression of a fluorophore further increases this phototoxicity (i.e., it acts as a photosensitizer). The dose dependence of this toxicity is complicated, with the toxicity depending not only on the total dose, but also on the intensity with which it is delivered (Carlton *et al.*, 2010; Tinevez *et al.*, 2012; Magidson and Khodjakov, 2013; Wäldchen *et al.*, 2015). This phototoxicity should be considered to ensure that it does not affect or confound the process being measured.

## EFFECT OF TAGS ON PROTEIN FUNCTION

When fluorescently tagging a protein, one must also consider the effect of the tag on the protein. Although it is impossible to predict whether a particular tag will be deleterious to a tagged protein,

some fluorescent tags suffer from problematic aggregation. Because many fluorescent tags and all intrinsically fluorescent proteins have been engineered from dimeric or tetrameric proteins, these tags often retain some residual aggregation tendency. This can result in tagged proteins aggregating and failing to fulfill their normal cellular functions. Assays have been developed to assess this aggregation (Costantini *et al.*, 2012; Jiang *et al.*, 2017). One that has been applied to many intrinsically fluorescent proteins measures the amount of whorl-like endoplasmic reticulum (ER) structures produced when a tag is fused to an ER membrane protein. These structures result from aggregation of the tagged protein and seem to be a sensitive indicator of aggregation and to correlate with how often the tag affects the function of other fusion proteins (Costantini *et al.*, 2012; Cranfill *et al.*, 2016). A lack of aggregation in these assays does not guarantee that a tagged protein will be fully functional, but proteins that aggregate strongly in these assays are unlikely to yield successful fusions.

## PHOTOCHROMIC TAGS

Fluorescent tags can be used to make measurements beyond the abundance and location of a tagged molecule. Many tags are photochromic, meaning that their fluorescence properties can be switched by light exposure. For example, a number of proteins are green-to-red photoconvertible, meaning that when synthesized, they fluoresce green, but they can be converted into a red fluorescent state by a brief pulse of near-UV light. Photoswitchable proteins can be switched from nonfluorescent to fluorescent (and back, in some cases) by light. These proteins can be used to track the movement of a subset of the tagged protein by exposing a region of the cell to UV, to carry out pulse-chase experiments by converting all of the protein at a specific time, and for superresolution microscopy. A complete discussion of photochromic molecules is beyond the scope of this review (for more information, see Zhou and Lin, 2013; Adam *et al.*, 2014; Matsuda and Nagai, 2014; Acharya *et al.*, 2017).

## REPORTER MOLECULES

As mentioned earlier, fluorescent proteins have been made to report on pH. Many other biosensors based on fluorescent proteins have been constructed as well. These range from sensors for other ions, such as calcium or zinc, to molecules that specifically respond to phosphorylation, proteolysis, or the activation state of molecules such as G-proteins. These reporters are typically constructed in one of two ways. A sensor element can be inserted into a (potentially circularly permuted) fluorescent protein such that binding of the analyte to the sensor changes the fluorescence intensity or spectrum of the fluorescent protein. Alternatively, two fluorescent proteins can be fused so that they undergo energy transfer. A sensor element between them changes the conformation of the complex on analyte binding, changing the energy transfer efficiency and hence the measured spectrum or fluorescence lifetime. Enzymatic processes such as proteolysis and protein degradation can be followed as well—proteolysis, by cleavage of a linker between two fluorescent proteins undergoing energy transfer or by cleavage of a linker between a fluorescent protein and a localization domain, leading to loss of localization; and protein degradation, by fusing a degradation domain to the fluorescent protein; this has been used to make cell cycle progress reporters (Sakaue-Sawano *et al.*, 2008; Bajar *et al.*, 2016a; for general reviews of reporter molecules, see Hochreiter *et al.*, 2015; Germond *et al.*, 2016).

The abundance and localization of small molecules can also be detected by using a sensor element to localize a fluorescent protein to specific molecules. For example, a number of specific lipid-binding proteins have been used to image the localization of those lipids (Halet, 2005), and similar approaches can be imagined for other molecules.

## TAGGING OF NUCLEIC ACIDS

Several systems exist for tagging RNAs by fusion of an RNA sequence that recruits a tagged RNA-binding protein (Lampasona and Czaplinski, 2016). Typically, these systems involve fusions of multiple RNA hairpins to the target RNA and coexpression of a fluorescent protein fused to a protein that specifically binds these hairpins. Because the fluorescent protein fusion is fluorescent regardless of whether it is bound to the RNA, a nuclear localization sequence is typically included as well so that unbound protein is sequestered in the nucleus. This reduces the cytoplasmic background, so that the RNA-bound fluorescent protein that is trafficked to the cytoplasm can be readily detected.

An alternative approach to RNA tagging is the engineering of RNA aptamers that bind an exogenous fluorophore (akin to extrinsically fluorescent proteins; Dolgosheina and Unrau, 2016; Ouellet, 2016). These provide an RNA-only tagging system, with no protein partner required. A wide variety of aptamers that bind a number of different fluorophores have been made (Table 2). In particular, a number of green fluorescent aptamers have been made that bind a fluorophore, DFHBI, related to the GFP fluorophore. DFHBI is nonfluorescent in water but becomes highly fluorescent on binding the aptamer (i.e., it lights up), resulting in high-contrast labeling. The best-performing aptamer appears to be F30Broccoli (Filonov *et al.*, 2015), a 105-nucleotide sequence that can be multimerized for increased brightness. Changing the fluorophore structure gives spectra similar to those of GFP or YFP (Song *et al.*, 2014). Other light-up aptamers that result in orange or far-red fluorescence have been developed, but these are less widely used (Babendure *et al.*, 2003; Dolgosheina *et al.*, 2014). A different approach to producing a light-up aptamer is to bind and sequester a contact quencher covalently fused to a fluorophore, enabling easy switching of spectral properties by switching the fluorophore (Arora *et al.*, 2015). Finally, there is an aptamer that binds benzylguanine derivatives, enabling the use of SNAP-tag reagents to label RNAs, although these need to be washed out of the cell because they are fluorescent when unbound (Xu *et al.*, 2016). All of the aptamers described here require supplementation with an exogenous fluorophore. In general, the development of fluorescent RNA aptamers lags behind the development of fluorescent proteins, although rapid progress is being made.

Although DNA-binding dyes such as 4′,6-diamidino-2-phenylindole or propidium iodide have been known for many years, they have little sequence specificity. Marking specific DNA loci was first done by introducing arrays of bacterial operator sites along with a fluorescent protein fused to the cognate binding domain. Of these, the most widely used systems are LacO (Robinett *et al.*, 1996) and TetR (Michaelis *et al.*, 1997). However, these techniques require introduction of large pieces of DNA (5–10 kb), potentially changing the properties of the DNA sequence under study. More recently, DNA targeting methods such as TALEs and the CRISPR-Cas9 system have been used to label arbitrary DNA sequences in mammalian cells. The CRISPR-Cas9 system can be used to target a specific genomic locus by coexpressing catalytically dead Cas9 (dCas9) fused to a fluorescent protein with a set of ~30 guide RNAs that cover the region to be targeted (Chen *et al.*, 2013). Multicolor imaging can be performed with the CRISPR-Cas9 system as well, either by tagging guide RNAs for different loci with sequences that recruit fluorescent protein fusions (Shao *et al.*, 2016) or by using orthogonal

CRISPR-Cas9 systems from different organisms (Chen *et al.*, 2016b). For a review of DNA-targeting approaches, see Chen *et al.* (2016a).

## FUTURE DEVELOPMENTS

Fluorescent proteins are now more than two decades old and are becoming a mature technology, although rapid development is ongoing, as evidenced by the recent publication of mScarlet (Bindels *et al.*, 2017), probably the brightest and best-behaved red fluorescent protein to date. Near-infrared and infrared fluorescent proteins still perform less well than their visible counterparts, and we can expect to see rapid continued development in improving brightness, increasing spectral diversity, and reducing the dependence on supplementation with fluorophores. In contrast to fluorescent proteins, RNA-tagging approaches are less well developed. I expect to see continued development of fluorescent RNA aptamers for RNA tagging. In principle, there is no reason that the brightness and spectral range of these proteins should not rival that of fluorescent proteins.

As in the rest of biology, CRISPR-Cas9 systems are having a major effect on fluorescent tagging approaches. CRISPR-Cas9 has already been modified into a DNA-tagging system in which fluorescently tagged, catalytically dead Cas9 is recruited to a specific locus by a panel of guide RNAs. A protein-tagging system amenable to CRISPR-Cas9 knock-in has also been developed (Leonetti *et al.*, 2016). In this system, GFP or mCherry is split into two pieces: one corresponds to the 11th β-strand and the other to β-strands 1–10. Separately, they are nonfluorescent, but when coexpressed, they bind and form a fluorescent GFP molecule. The 11th β-strand is only 16 amino acids long—small enough to be encoded with a commercial oligonucleotide. Because no cloning is needed, this system enables rapid tagging of genes in a high-throughput manner. Owing to its simplicity, this approach is likely to become increasingly popular. There is no reason it could not be extended to additional fluorescent tags, although some protein engineering will likely be required. I expect that this and similar systems, yet to be developed, will have a major effect on biological imaging in the future.

## CONCLUSION

Over the past 20 years, the ready availability of multicolor genetically encoded fluorescent tags has revolutionized live-cell imaging. New tags continue to be developed that are brighter, expand the available spectral range, and are better tolerated by fusions. More recently, tools for tagging nucleic acids have become widely available. Combined with recent developments in advanced microscopy techniques for high-speed, high-resolution of live cells and organisms, these probes enable increasingly detailed studies of biological processes.


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