Genetically encoded fluorescent tags

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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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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

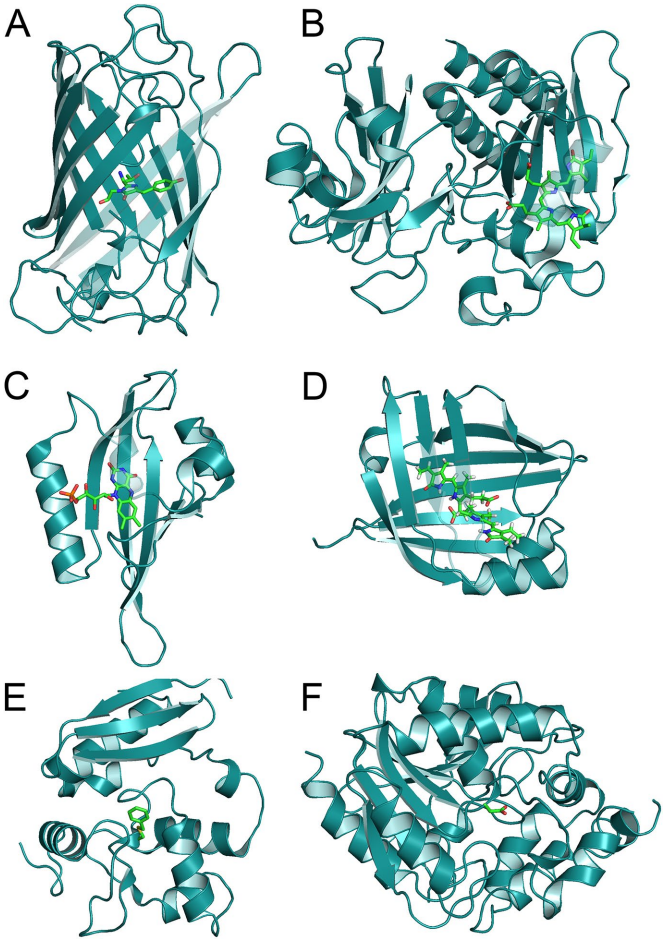

FIGURE 1: Structures of representative fluorescent tags. In all cases, the protein backbone is shown as a cartoon, and the fluorophore is drawn as a stick model (green, carbon; blue, nitrogen; red, oxygen; orange, phosphorus). In some cases, parts of the protein cartoon have been made transparent to 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	λ_{ex} (nm)	λ_{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	λ_{ex} (nm)	λ_{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. λ_{ex} and λ_{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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REFERENCES

Acharya A, Bogdanov AM, Grigorenko BL, Bravaya KB, Nemukhin AV, Lukyanov KA, Krylov AI (2017). Photoinduced chemistry in fluorescent proteins: curse or blessing? Chem Rev 117, 758–795.

Adam V, Berardozzi R, Byrdin M, Bourgeois D (2014). Phototransformable fluorescent proteins: future challenges. Curr Opin Chem Biol 20, 92–102.

Ai H, Olenych SG, Wong P, Davidson MW, Campbell RE (2008). Hue-shifted monomeric variants of Clavularia cyan fluorescent protein: identification of the molecular determinants of color and applications in fluorescence imaging. BMC Biol 6, 13.

Alieva NO, Konzen KA, Field SF, Meleshkevitch EA, Hunt ME, Beltran-Ramirez V, Miller DJ, Wiedenmann J, Salih A, Matz MV (2008). Diversity and evolution of coral fluorescent proteins. PLoS One 3, e2680.

Arora A, Sunbul M, Jäschke A (2015). Dual-colour imaging of RNAs using quencher- and fluorophore-binding aptamers. Nucleic Acids Res 43, e144.

Babendure JR, Adams SR, Tsien RY (2003). Aptamers switch on fluorescence of triphenylmethane dyes. J Am Chem Soc 125, 14716–14717.

Baird GS, Zacharias DA, Tsien RY (2000). Biochemistry, mutagenesis, and oligomerization of DsRed, a red fluorescent protein from coral. Proc Natl Acad Sci USA 97, 11984–11989.

Bajar BT, Lam AJ, Badiee RK, Oh Y-H, Chu J, Zhou XX, Kim N, Kim BB, Chung M, Yablonovitch AL, *et al.* (2016a). Fluorescent indicators for simultaneous reporting of all four cell cycle phases. Nat Methods 13, 993–996.

Bajar BT, Wang ES, Lam AJ, Kim BB, Jacobs CL, Howe ES, Davidson MW, Lin MZ, Chu J (2016b). Improving brightness and photostability of green and red fluorescent proteins for live cell imaging and FRET reporting. Sci Rep 6, 20889.

Bindels DS, Haarbosch L, van Weeren L, Postma M, Wiese KE, Mastop M, Aumonier S, Gotthard G, Royant A, Hink MA, *et al.* (2017). mScarlet: a bright monomeric red fluorescent protein for cellular imaging. Nat Methods 14, 53–56.

Bruchez MP (2015). Dark dyes-bright complexes: fluorogenic protein labeling. Curr Opin Chem Biol 27, 18–23.

Buckley AM, Petersen J, Roe AJ, Douce GR, Christie JM (2015). LOV-based reporters for fluorescence imaging. Curr Opin Chem Biol 27, 39–45.

Carlton PM, Boulanger J, Kervrann C, Sibarita J-B, Salamero J, Gordon-Messer S, Bressan D, Haber JE, Haase S, Shao L, *et al.* (2010). Fast live simultaneous multiwavelength four-dimensional optical microscopy. Proc Natl Acad Sci USA 107, 16016–16022.

Chalfie M (2009). GFP: lighting up life (Nobel Lecture). Angew Chem Int Ed Engl 48, 5603–5611.

Chapman S, Faulkner C, Kaiserli E, Garcia-Mata C, Savenkov EI, Roberts AG, Oparka KJ, Christie JM (2008). The photoreversible fluorescent protein iLOV outperforms GFP as a reporter of plant virus infection. Proc Natl Acad Sci USA 105, 20038–20043.

Chen B, Gilbert LA, Cimini BA, Schnitzbauer J, Zhang W, Li G-W, Park J, Blackburn EH, Weissman JS, Qi LS, *et al.* (2013). Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. Cell 155, 1479–1491.

Chen B, Guan J, Huang B (2016a). Imaging specific genomic DNA in living cells. Annu Rev Biophys 45, 1–23.

Chen B, Hu J, Almeida R, Liu H, Balakrishnan S, Covill-Cooke C, Lim WA, Huang B (2016b). Expanding the CRISPR imaging toolset with Staphylococcus aureus Cas9 for simultaneous imaging of multiple genomic loci. Nucleic Acids Res 44, e75.

Chen Z, Jing C, Gallagher SS, Sheetz MP, Cornish VW (2012). Second-generation covalent TMP-Tag for live cell imaging. J Am Chem Soc 134, 13692–13699.

Christie JM, Hitomi K, Arvai AS, Hartfield KA, Mettlen M, Pratt AJ, Tainer JA, Getzoff ED (2012). Structural tuning of the fluorescent protein iLOV for improved photostability. J Biol Chem 287, 22295–22304.

Chu J, Haynes RD, Corbel SY, Li P, González-González E, Burg JS, Ataie NJ, Lam AJ, Cranfill PJ, Baird MA, *et al.* (2014). Non-invasive intravital imaging of cellular differentiation with a bright red-excitable fluorescent protein. Nat Methods 11, 572–578.

Chudakov DM, Matz MV, Lukyanov S, Lukyanov KA (2010). Fluorescent proteins and their applications in imaging living cells and tissues. Physiol Rev 90, 1103–1163.

Costantini LM, Fossati M, Francolini M, Snapp EL (2012). Assessing the tendency of fluorescent proteins to oligomerize under physiologic conditions. Traffic 13, 643–649.

Cranfill PJ, Sell BR, Baird MA, Allen JR, Lavagnino Z, de Gruiter HM, Kremers G-J, Davidson MW, Ustione A, Piston DW (2016). Quantitative assessment of fluorescent proteins. Nat Methods 13, 557–562.

Cutrale F, Trivedi V, Trinh LA, Chiu C-L, Choi JM, Artiga MS, Fraser SE (2017). Hyperspectral phasor analysis enables multiplexed 5D in vivo imaging. Nat Methods 14, 149–152.

Dolgosheina EV, Jeng SCY, Panchapakesan SSS, Cojocaru R, Chen PSK, Wilson PD, Hawkins N, Wiggins PA, Unrau PJ (2014). RNA mango aptamer-fluorophore: a bright, high-affinity complex for RNA labeling and tracking. ACS Chem Biol 9, 2412–2420.

Dolgosheina EV, Unrau PJ (2016). Fluorophore-binding RNA aptamers and their applications. Wiley Interdiscip Rev RNA 7, 843–851.

Drepper T, Eggert T, Circolone F, Heck A, Krauss U, Guterl J-K, Wendorff M, Losi A, Gärtner W, Jaeger K-E (2007). Reporter proteins for in vivo fluorescence without oxygen. Nat Biotechnol 25, 443–445.

Filonov GS, Kam CW, Song W, Jaffrey SR (2015). In-gel imaging of RNA processing using broccoli reveals optimal aptamer expression strategies. Chem Biol 22, 649–660.
Fisher AC, DeLisa MP (2008). Laboratory evolution of fast-folding green fluorescent protein using secretory pathway quality control. PLoS One 3, e2351.
Gaietta G, Deerinck TJ, Adams SR, Bouwer J, Tour O, Laird DW, Sosinsky GE, Tsien RY, Ellisman MH (2002). Multicolor and electron microscopic imaging of connexin trafficking. Science 296, 503–507.
Gautier A, Juillerat A, Heinis C, Corrêa IR, Kindermann M, Beaufils F, Johnsson K (2008). An engineered protein tag for multiprotein labeling in living cells. Chem Biol 15, 128–136.
Germond A, Fujita H, Ichimura T, Watanabe TM (2016). Design and development of genetically encoded fluorescent sensors to monitor intracellular chemical and physical parameters. Biophys Rev 8, 121–138.
Goedhart J, von Stetten D, Noirclerc-Savoye M, Lelimousin M, Joosen L, Hink MA, van Weeren L Jr, Gadella TWJ, Royant A (2012). Structure-guided evolution of cyan fluorescent proteins towards a quantum yield of 93%. Nat Commun 3, 751.
Griesbeck O, Baird GS, Campbell RE, Zacharias DA, Tsien RY (2001). Reducing the environmental sensitivity of yellow fluorescent protein. mechanism and applications. J Biol Chem 276, 29188–29194.
Griffin BA, Adams SR, Tsien RY (1998). Specific covalent labeling of recombinant protein molecules inside live cells. Science 281, 269–272.
Grimm JB, English BP, Chen J, Slaughter JP, Zhang Z, Revyakin A, Patel R, Macklin JJ, Normanno D, Singer RH, *et al*. (2015). A general method to improve fluorophores for live-cell and single-molecule microscopy. Nat Methods 12, 244–250, 3 p following 250.
Halet G (2005). Imaging phosphoinositide dynamics using GFP-tagged protein domains. Biol Cell 97, 501–518.
Heppert JK, Dickinson DJ, Pani AM, Higgins CD, Steward A, Ahringer J, Kuhn JR, Goldstein B (2016). Comparative assessment of fluorescent proteins for in vivo imaging in an animal model system. Mol Biol Cell 27, 3385–3394.
Hochreiter B, Garcia AP, Schmid JA (2015). Fluorescent proteins as genetically encoded FRET biosensors in life sciences. Sensors 15, 26281–26314.
Iizuka R, Yamagishi-Shirasaki M, Funatsu T (2011). Kinetic study of de novo chromophore maturation of fluorescent proteins. Anal Biochem 414, 173–178.
Jiang Y, Di Gregorio SE, Duennwald ML, Lajoie P (2017). Polyglutamine toxicity in yeast uncovers phenotypic variations between different fluorescent protein fusions. Traffic 18, 58–70.
Jing C, Cornish VW (2013). A fluorogenic TMP-tag for high signal-to-background intracellular live cell imaging. ACS Chem Biol 8, 1704–1712.
Kamiyama D, Sekine S, Barsi-Rhyne B, Hu J, Chen B, Gilbert LA, Ishikawa H, Leonetti MD, Marshall WF, Weissman JS, *et al*. (2016). Versatile protein tagging in cells with split fluorescent protein. Nat Commun 7, 11046.
Keppler A, Gendreizig S, Gronemeyer T, Pick H, Vogel H, Johnsson K (2003). A general method for the covalent labeling of fusion proteins with small molecules in vivo. Nat Biotechnol 21, 86–89.
Kuipers J, van Ham TJ, Kalicharan RD, Veenstra-Algra A, Sjollema KA, Dijk F, Schnell U, Giepmans BNG (2015). FLIPPER, a combinatorial probe for correlated live imaging and electron microscopy, allows identification and quantitative analysis of various cells and organelles. Cell Tissue Res 360, 61–70.
Kumagai A, Ando R, Miyatake H, Greimel P, Kobayashi T, Hirabayashi Y, Shimogori T, Miyawaki A (2013). A bilirubin-inducible fluorescent protein from eel muscle. Cell 153, 1602–1611.
Labas YA, Gurskaya NG, Yanushevich YG, Fradkov AF, Lukyanov KA, Lukyanov SA, Matz MV (2002). Diversity and evolution of the green fluorescent protein family. Proc Natl Acad Sci USA 99, 4256–4261.
Lam SS, Martell JD, Kamer KJ, Deerinck TJ, Ellisman MH, Mootha VK, Ting AY (2015). Directed evolution of APEX2 for electron microscopy and proximity labeling. Nat Methods 12, 51–54.
Lampasona AA, Czaplinski K (2016). RNA voyeurism: a coming of age story. Methods 98, 10–17.
Laviv T, Kim BB, Chu J, Lam AJ, Lin MZ, Yasuda R (2016). Simultaneous dual-color fluorescence lifetime imaging with novel red-shifted fluorescent proteins. Nat Methods 13, 989–992.
Lee S, Lim WA, Thorn KS (2013). Improved blue, green, and red fluorescent protein tagging vectors for S. cerevisiae. PLoS One 8, e67902.
Leonetti MD, Sekine S, Kamiyama D, Weissman JS, Huang B (2016). A scalable strategy for high-throughput GFP tagging of endogenous human proteins. Proc Natl Acad Sci USA 113, E3501–E3508.
Livet J, Weissman TA, Kang H, Draft RW, Lu J, Bennis RA, Sanes JR, Lichtman JW (2007). Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system. Nature 450, 56–62.
Los GV, Encell LP, McDougall MG, Hartzell DD, Karassina N, Zimprich C, Wood MG, Learish R, Ohana RF, Urh M, *et al*. (2008). HaloTag: a novel protein labeling technology for cell imaging and protein analysis. ACS Chem Biol 3, 373–382.
Magidson V, Khodjakov A (2013). Circumventing photodamage in live-cell microscopy. Methods Cell Biol 114, 545–560.
Markwardt ML, Kremers G-J, Kraft CA, Ray K, Cranfill PJC, Wilson KA, Day RN, Wachter RM, Davidson MW, Rizzo MA (2011). An improved cerulean fluorescent protein with enhanced brightness and reduced reversible photoswitching. PLoS One 6, e17896.
Martell JD, Deerinck TJ, Sancak Y, Poulos TL, Mootha VK, Sosinsky GE, Ellisman MH, Ting AY (2012). Engineered ascorbate peroxidase as a genetically encoded reporter for electron microscopy. Nat Biotechnol 30, 1143–1148.
Martin BR, Giepmans BNG, Adams SR, Tsien RY (2005). Mammalian cell–based optimization of the biarsenical-binding tetracysteine motif for improved fluorescence and affinity. Nat Biotechnol 23, 1308–1314.
Matsuda T, Nagai T (2014). Quantitative measurement of intracellular protein dynamics using photobleaching or photoactivation of fluorescent proteins. Microscopy 63, 403–408.
Merzlyak EM, Goedhart J, Shcherbo D, Bulina ME, Shcheglov AS, Fradkov AF, Gaintzeva A, Lukyanov KA, Lukyanov S, Gadella TWJ, *et al*. (2007). Bright monomeric red fluorescent protein with an extended fluorescence lifetime. Nat Methods 4, 555–557.
Michaelis C, Ciosk R, Nasmyth K (1997). Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. Cell 91, 35–45.
Miesenböck G, De Angelis DA, Rothman JE (1998). Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. Nature 394, 192–195.
Miller LW, Cai Y, Sheetz MP, Cornish VW (2005). In vivo protein labeling with trimethoprim conjugates: a flexible chemical tag. Nat Methods 2, 255–257.
Mollwitz B, Brunk E, Schmitt S, Pojer F, Bannwarth M, Schiltz M, Rothlisberger U, Johnsson K (2012). Directed evolution of the suicide protein O^6-alkylguanine-DNA alkyltransferase for increased reactivity results in an alkylated protein with exceptional stability. Biochemistry 51, 986–994.
Mukherjee A, Weyant KB, Agrawal U, Walker J, Cann IKO, Schroeder CM (2015). Engineering and characterization of new LOV-based fluorescent proteins from chlamydomonas reinhardtii and vaucheria frigida. ACS Synth Biol 4, 371–377.
Nagai T, Ibata K, Park ES, Kubota M, Mikoshiba K, Miyawaki A (2002). A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. Nat Biotechnol 20, 87–90.
Newman J, Peat TS, Richard R, Kan L, Swanson PE, Affholter JA, Holmes IH, Schindler JF, Unkefer CJ, Terwilliger TC (1999). Haloalkane dehalogenases: structure of a rhodococcus enzyme. Biochemistry 38, 16105–16114.
Ormö M, Cubitt AB, Kallio K, Gross LA, Tsien RY, Remington SJ (1996). Crystal structure of the Aequorea victoria green fluorescent protein. Science 273, 1392–1395.
Ouellet J (2016). RNA fluorescence with light-up aptamers. Front Chem 4, 29.
Pédelacq J-D, Cabantous S, Tran T, Terwilliger TC, Waldo GS (2006). Engineering and characterization of a superfolder green fluorescent protein. Nat Biotechnol 24, 79–88.
Ren H, Yang B, Ma C, Hu YS, Wang PG, Wang L (2016). Cysteine sulfoxidation increases the photostability of red fluorescent proteins. ACS Chem Biol 11, 2679–2684.
Robinett CC, Straight A, Li G, Willhelm C, Sudlow G, Murray A, Belmont AS (1996). In vivo localization of DNA sequences and visualization of large-scale chromatin organization using lac operator/repressor recognition. J Cell Biol 135, 1685–1700.
Rodriguez EA, Campbell RE, Lin JY, Lin MZ, Miyawaki A, Palmer AE, Shu X, Zhang J, Tsien RY (2016a). The growing and glowing toolbox of fluorescent and photoactive proteins. Trends Biochem Sci 42, 111–129.
Rodriguez EA, Tran GN, Gross LA, Crisp JL, Shu X, Lin JY, Tsien RY (2016b). A far-red fluorescent protein evolved from a cyanobacterial phycobiliprotein. Nat Methods 13, 763–769.
Sakaue-Sawano A, Kurokawa H, Morimura T, Hanyu A, Hama H, Osawa H, Kashiwagi S, Fukami K, Miyata T, Miyoshi H, *et al*. (2008). Visualizing spatiotemporal dynamics of multicellular cell-cycle progression. Cell 132, 487–498.

Shaner NC, Campbell RE, Steinbach PA, Giepmans BNG, Palmer AE, Tsien RY (2004). Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. Nat Biotechnol 22, 1567–1572.
Shaner NC, Lambert GG, Chammas A, Ni Y, Cranfill PJ, Baird MA, Sell BR, Allen JR, Day RN, Israelsson M, *et al*. (2013). A bright monomeric green fluorescent protein derived from Branchiostoma lanceolatum. Nat Methods 10, 407–409.
Shaner NC, Lin MZ, McKeown MR, Steinbach PA, Hazelwood KL, Davidson MW, Tsien RY (2008). Improving the photostability of bright monomeric orange and red fluorescent proteins. Nat Methods 5, 545–551.
Shao S, Zhang W, Hu H, Xue B, Qin J, Sun C, Sun Y, Wei W, Sun Y (2016). Long-term dual-color tracking of genomic loci by modified sgRNAs of the CRISPR/Cas9 system. Nucleic Acids Res 44, e86.
Shcherbakova DM, Baloban M, Emelyanov AV, Brenowitz M, Guo P, Verkhusha VV (2016). Bright monomeric near-infrared fluorescent proteins as tags and biosensors for multiscale imaging. Nat Commun 7, 12405.
Shcherbakova DM, Hink MA, Joosen L, Gadella TWJ, Verkhusha VV (2012). An orange fluorescent protein with a large stokes shift for single-excitation multicolor FCCS and FRET imaging. J Am Chem Soc 134, 7913–7923.
Shcherbakova DM, Verkhusha VV (2013). Near-infrared fluorescent proteins for multicolor in vivo imaging. Nat Methods 10, 751–754.
Shcherbo D, Murphy CS, Ermakova GV, Solovieva EA, Chepurnykh TV, Shcheglov AS, Verkhusha VV, Pletnev VZ, Hazelwood KL, Roche PM, *et al*. (2009). Far-red fluorescent tags for protein imaging in living tissues. Biochem J 418, 567–574.
Shemiakina II, Ermakova GV, Cranfill PJ, Baird MA, Evans RA, Souslova EA, Staroverov DB, Gorokhovatsky AY, Putintseva EV, Gorodnicheva TV, *et al*. (2012). A monomeric red fluorescent protein with low cytotoxicity. Nat Commun 3, 1204.
Shu X, Lev-Ram V, Deerinck TJ, Qi Y, Ramko EB, Davidson MW, Jin Y, Ellisman MH, Tsien RY (2011). A genetically encoded tag for correlated light and electron microscopy of intact cells, tissues, and organisms. PLoS Biol 9, e1001041.
Song W, Strack RL, Svensen N, Jaffrey SR (2014). Plug-and-play fluorophores extend the spectral properties of spinach. J Am Chem Soc 136, 1198–1201.
Subach OM, Cranfill PJ, Davidson MW, Verkhusha VV (2011). An enhanced monomeric blue fluorescent protein with the high chemical stability of the chromophore. PLoS One 6, e28674.
Sun X, Zhang A, Baker B, Sun L, Howard A, Buswell J, Maurel D, Masharina A, Johnsson K, Noren CJ, *et al*. (2011). Development of SNAP-tag fluorogenic probes for wash-free fluorescence imaging. Chembiochem 12, 2217–2226.
Szent-Gyorgyi C, Schmidt BF, Schmidt BA, Creeger Y, Fisher GW, Zakel KL, Adler S, Fitzpatrick JAJ, Woolford CA, Yan Q, *et al*. (2008). Fluorogen-activating single-chain antibodies for imaging cell surface proteins. Nat Biotechnol 26, 235–240.
Tanenbaum ME, Gilbert LA, Qi LS, Weissman JS, Vale RD (2014). A protein-tagging system for signal amplification in gene expression and fluorescence imaging. Cell 159, 635–646.
Thorn K (2016). A quick guide to light microscopy in cell biology. Mol Biol Cell 27, 219–222.
Tinevez J-Y, Dragavon J, Baba-Aissa L, Roux P, Perret E, Canivet A, Galy V, Shorte S (2012). A quantitative method for measuring phototoxicity of a live cell imaging microscope. Methods Enzymol 506, 291–309.
Tsien RY (1998). The green fluorescent protein. Annu Rev Biochem 67, 509–544.
Tsien RY (2009). Constructing and exploiting the fluorescent protein paintbox (Nobel Lecture). Angew Chem Int Ed Engl 48, 5612–5626.
Tsutsui H, Karasawa S, Okamura Y, Miyawaki A (2008). Improving membrane voltage measurements using FRET with new fluorescent proteins. Nat Methods 5, 683–685.
Wäldchen S, Lehmann J, Klein T, van de Linde S, Sauer M (2015). Light-induced cell damage in live-cell super-resolution microscopy. Sci Rep 5, 15348.
Xu J, Carrocci TJ, Hoskins AA (2016). Evolution and characterization of a benzylguanine-binding RNA aptamer. Chem Commun 52, 549–552.
Yang J, Wang L, Yang F, Luo H, Xu L, Lu J, Zeng S, Zhang Z (2013). mBeRFP, an improved large Stokes shift red fluorescent protein. PLoS One 8, e64849.
Yang TT, Cheng L, Kain SR (1996). Optimized codon usage and chromophore mutations provide enhanced sensitivity with the green fluorescent protein. Nucleic Acids Res 24, 4592–4593.
Yu D, Baird MA, Allen JR, Howe ES, Klassen MP, Reade A, Makhijani K, Song Y, Liu S, Murthy Z, *et al*. (2015). A naturally monomeric infrared fluorescent protein for protein labeling in vivo. Nat Methods 12, 763–765.
Yu D, Gustafson WC, Han C, Lafaye C, Noirclerc-Savoye M, Ge W-P, Thayer DA, Huang H, Kornberg TB, Royant A, *et al*. (2014). An improved monomeric infrared fluorescent protein for neuronal and tumour brain imaging. Nat Commun 5, 3626.
Zacharias DA, Violin JD, Newton AC, Tsien RY (2002). Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. Science 296, 913–916.
Zapata-Hommer O, Griesbeck O (2003). Efficiently folding and circularly permuted variants of the Sapphire mutant of GFP. BMC Biotechnol 3, 5.
Zhou XX, Lin MZ (2013). Photoswitchable fluorescent proteins: ten years of colorful chemistry and exciting applications. Curr Opin Chem Biol 17, 682–690.
Zimmermann T (2005). Spectral imaging and linear unmixing in light microscopy. Adv Biochem Eng Biotechnol 95, 245–265.