Fluorescent proteins at a glance

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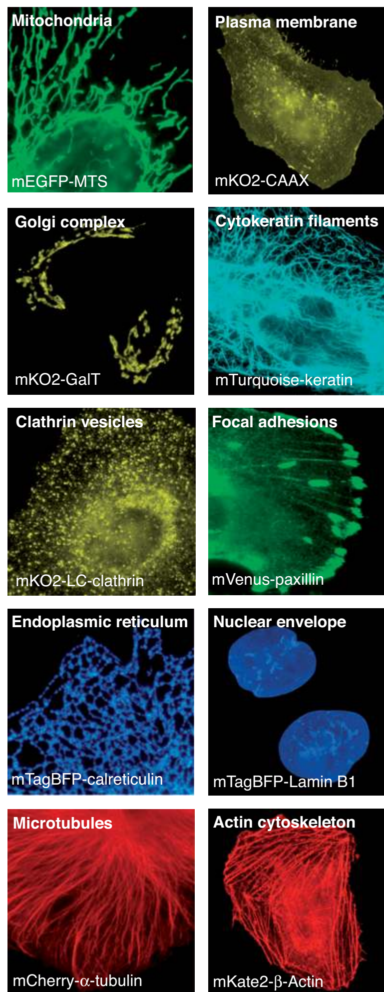
There was an error published in *J. Cell Sci*. **124**, 157-160.

During the course of creating the accompanying poster, pseudocolored fluorescent protein fusion images from previous publications were used to define the layout. Many of these images were inadvertently not replaced with a similar image of the correct fusion in the final published version. This affects the following images from the "Fluorescent protein localization" panel of the poster (note that the identities of the placeholder images are denoted in parentheses): mEGFP-MTS (mWasabi-MTS); mKO2-CAAX (TagRFP-T-annexin); mKO2-GalT (TagRFP-T-GalT); mTurquoise-keratin (mTFP1-keratin); mKO2-LC-clathrin (TagRFP-T-clathrin); mVenus-paxillin (mWasabi-paxillin); mTagBFP-calreticulin (mTFP1-calreticulin). In assembling the correction, the following raw images could not be located and thus shown here are newly acquired images: mTagBFP-lamin B1; mCherry-α-tubulin; mKate2-β-actin.

The correct images and labels are as follows:



The authors apologise for this error.

A corrected version of the poster is available for downloading here. http://jcs.biologists.org/content/124/2/157/suppl/DC2

A similar error arose in a previous publication, and an inquiry and investigation have been conducted by Michael Davidson's institution. It was determined that the problems with the figures were not the result of deliberate alterations of data to support the scientific conclusions of the publications.

Fluorescent proteins at a glance

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Introduction to fluorescent proteins

The original green fluorescent protein (GFP) was discovered back in the early 1960s when researchers studying the bioluminescent properties of the *Aequorea victoria* jellyfish isolated a blue-light-emitting bioluminescent protein called aequorin together with another protein that was eventually named the green-fluorescent protein (Shimomura et al., 1962). Aequorin and GFP work together in the light organs of *A. victoria* to convert Ca^{2+}-induced luminescent signals into the green luminescence that is characteristic of the species (Chalfie and Kain, 2006). After GFP was cloned (Prasher et al., 1992), it was first used for tracking gene expression in bacteria and the sensory neurons of the nematode *C. elegans* (Chalfie et al., 1994). Jellyfish-derived GFP has since been engineered to produce a vast number of useful blue, cyan and yellow mutants, and fluorescent proteins from a variety of other species have also been identified, resulting in further expansion of the available color palette into the orange, red and far-red spectral regions (Matz et al., 1999; Shaner et al., 2004; Shaner et al., 2008; Shcherbo et al., 2009). Together, these highly useful genetically encoded probes are broadly referred to as fluorescent proteins (FPs) (Davidson and Campbell, 2009; Rizzo et al., 2010; Shaner et al., 2007).

In this article and the accompanying poster, we will describe some of the general properties of FPs that are important to their function. We will also provide examples of successful mutagenesis that has been used to improve the use of these proteins for live-cell imaging, particularly for mammalian studies. Particular emphasis will be given to some of the most popular and/or best performing FPs in various spectral regions (blue, cyan, green, yellow, orange, red and far-red). Finally, we will briefly present some other applications of FPs, as well as outline anticipated future improvements. This short article is meant to provide a flavor of the

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CHROMA

Excitation and emission spectral properties of the brightest fluorescent proteins

Excitation intensity; Emission intensity; Wavelength (nm)

Key: TagBFP, mTurquoise, EGFP, mVenus, mKO2, mApple, mCherry, mKate2

Fluorescent protein properties

Protein (acronym)	Excitation maximum (nm)	Emission maximum (nm)	Extinction coefficient $\times 10^{-3}$ ($M^{-1}cm^{-1}$)	Quantum yield	Relative brightness (% of EGFP)
mTagBFP	399	456	52.0	0.63	98
mTurquoise	434	474	30.0	0.84	75
mEGFP	488	507	56.0	0.60	100
mVenus	515	528	92.2	0.57	156
mKO2	551	565	63.8	0.62	118
mApple	568	592	75.0	0.49	109
mCherry	587	610	72.0	0.22	47
mKate2	588	633	62.5	0.40	74

β-barrel motif

α-helix; N-terminus; C-terminus; β-barrel; Chromophore; β-sheet; Loop; ~4 nm; ~3 nm

Critical mutations

Mutation	Properties
S30R	Increases folding rate, enhances protein stability
F64L	Accelerates chromophore formation
Q69M	Improves chloride and pH resistance, photostability and folding
S72A	Faster folding rate, stabilizes protein
S147P	Faster maturation rate, located near chromophore
N149K	Faster folding rate, stabilizes protein
V163A	Reduces hydrophobicity, no effect on folding rate
I167T	Reduced thermosensitivity, faster maturation rate

Multi-color imaging using fluorescent protein fusions

2-color: LLC-PK1 (pig kidney) cells expressing mEGFP fused to α-tubulin (green) and mApple fused to histone H2B (red)

3-color: HeLa (human carcinoma) cells expressing mTurquoise fused to a Golgi-targeting peptide (cyan), mVenus fused to a nuclear targeting signal (yellow), and mCherry fused to a mitochondrion-targeting peptide (red)

4-color: RK-13 (rabbit kidney) cells expressing mTagBFP fused to histone H2B (blue), mTurquoise fused to peroxisomal membrane protein (cyan), mEGFP fused to Lifeact (actin; green), and mCherry fused to pyruvate dehydrogenase (mitochondria; red)

5-color: HeLa cells expressing mTagBFP fused to histone H2B (blue), mTurquoise fused to peroxisomal membrane protein (cyan), mEGFP fused to calreticulin (ER; green), mKO2 fused to zyxin (focal adhesions; purple), and mKate2 fused to pyruvate dehydrogenase (mitochondria; red)

Fluorescent protein localization

Mitochondria: mEGFP-MTS; Intermediate filaments: mCherry-Vimentin; Gap junctions: mVenus-Cx43
Plasma membrane: mKO2-CAAX; Microtubule plus ends: mTurquoise-EB3; Lysosomes: mKate2-LAMP1
Peroxisomes: mApple-PMP; Tight junctions: mEGFP-VE Cadherin; Golgi complex: mKO2-GalT
Nuclear envelope: mTagBFP-Lamin B1; Actin cytoskeleton: mKate2-β-Actin; Cellular junctions: mEGFP-β-Catenin
Cytokeratin filaments: mTurquoise-keratin; Centromeres: mEGFP-CENPB; Clathrin vesicles: mKO2-LC-clathrin
Microtubules: mCherry-α-tubulin; Focal adhesions: mVenus-paxillin; Endoplasmic reticulum: mTagBFP-calreticulin

Abbreviations: CAAX, C-terminal amino acid sequence for farnesylation; CENPB, centromere protein B; Cx43, connexin 43; EB3, end-binding protein 3; GalT, N-terminal 59 amino acids of human β1,4-galactosyltransferase; LAMP1, lysosomal membrane glycoprotein 1; LC-clathrin, light-chain clathrin; MTS, mitochondria targeting signal; VE cadherin, vesicular epithelial cadherin.

Fluorescent Protein Source References: **mApple:** Shaner, N. C., et al. (2008). *Nature Methods* **5**, 545–551; **mCherry:** Shaner, N. C., et al. (2004). *Nature Biotechnol.* **22**, 1567–1572; **mEGFP:** Cormack, B. P., et al. (1996). *Gene* **173**, 33–38; **mKate2:** Shcherbo, D., et al. (2009). *Biochem. J.* **418**, 567–576; **mKO2:** Sakaue-Sawano, A., et al. (2008). *Cell* **132**, 487–498; **mTagBFP:** Subach, O. M., et al. (2008). *Chem. Biol.* **59**, 1116–1124; **mTurquoise:** Goedhart, J., et al. (2010). *Nature Meth.* **7**, 137–139; **mVenus:** Nagai, T., et al. (2002). *Nature Biotechnol.* **20**, 87–90.

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(See poster insert)

power and limitations of fluorescent protein technologies; comprehensive reviews of their biochemistry (Tsien, 1998), their uses and available probes (Day and Davidson, 2009; Rizzo et al., 2010) are available elsewhere.

General properties of fluorescent proteins

Regardless of the originating species or degree of genetic manipulation, all FPs are ~25 kD in size, which is large compared with organic fluorophores (such as fluorescein or Texas Red) with average sizes of around 1 kD. Despite their rather large size, FPs are beneficial for many applications, in particular for live-cell and whole-animal imaging. FPs are genetic labels and thus can be 'built in' using transgenic approaches. As labels are created within the cell, there is no need for labeling with exogenous agents, or the fixation and permeabilization procedures that are required for immunofluorescence. FPs can also be fused to their protein targets, so that they are expressed in a 1:1 ratio with the target molecule, a fact that, of course, is ideal for quantitative imaging (Patterson et al., 1997).

One of the most important points regarding FPs is that the entire protein structure is essential to the development and maintenance of its fluorescence (Remington, 2006). The FP structure consists of an extremely rigid β-barrel-fold comprising 11 β-sheets that surround a central α-helix (Ormo et al., 1996). In all of the jellyfish and coral FPs studied thus far, the principle chromophore is derived from only a few crucial amino acids that are located near the center of the β-barrel (see 'β-barrel motif' in the poster). However, unlike the amino acids of most soluble proteins, many of the interior amino acids in FPs are charged or polar. They bind numerous water molecules and lock them into rigid conformations inside the protein. Within the context of this specific environment, a reaction occurs between key FP amino acids to form an imidazole ring with extended conjugation (Tsien, 1998). The fluorescence of these proteins is highly dependent on the unique chemical environment surrounding the chromophore, as evidenced by the fact that synthetic chromophore analogs are devoid of fluorescence (Follenius-Wund et al., 2003). Changes to the local chromophore environment also produce dramatic variations in spectral characteristics, photostability, acid resistance and a variety of other physical properties.

The mechanism of chromophore formation is thought to be similar for every FP, regardless of the source (Remington, 2006). Examination of the amino acid sequences of over 100 naturally occurring chromophore variants from many species revealed that only four residues are absolutely conserved (Remington, 2006). The first residue is G67, which is crucial for cyclization of the chromophore through nucleophilic attack; consequently, any mutation of this amino acid completely obliterates chromophore formation. The second conserved residue is Y66, which is also involved in chromophore formation. However, mutagenesis studies show that any aromatic residue can replace Y66 (Heim et al., 1994), and it is therefore puzzling why this amino acid is so highly conserved in nature. The last two conserved amino acid residues are R96 and E222, both of which are catalytic residues that are positioned near the chromophore and essential to the maturation process. Several other residues near the chromophore, such as G20, G33, Gl91 and F130, are also conserved among FPs and are also thought to be involved in chromophore formation. As most of the other residues are not conserved, FPs can accommodate a high degree of modification to create proteins with different physical properties (Day and Davidson, 2009; Shaner et al., 2007).

Mutations that improve fluorescent proteins as imaging probes

Because of the unique β-barrel fold of fluorescent proteins, mutations of residues throughout the entire protein have the potential to significantly change their fluorescent properties. As is highlighted in the poster, the most striking result of such mutations is the wide range of different emission colors that is currently available, which greatly increases the usefulness of these proteins as molecular probes. However, most single mutations have a negative impact on the tight packing of the FP β-barrel and, therefore, result in greater environmental sensitivity and reduced brightness. Although some of these defects can be compensated for by additional mutations, derivative FPs are often less bright and/or more sensitive to the environment compared with the original protein. This phenomenon has been especially evident during the search for truly monomeric versions of the tetrameric red fluorescent protein of the coral *Discosoma* sp. (DsRed). Although several such monomeric variants have been generated, they are significantly less bright that the original DsRed (Shaner et al., 2004; Shaner et al., 2008).

To be able to use jellyfish-derived GFPs in mammalian systems, several properties of the original GFP had to be modified and such modifications are now found in all of the commonly used variants (see 'Critical mutations' in the poster). First, the maturation of the fluorescence was optimized for use at 37°C. Maturation of the wild-type GFP chromophore is efficient at temperatures lower than 28°C but increasing the temperature to 37°C substantially reduces the maturation rate and decreases fluorescence (Patterson et al., 1997). A single mutation of F64L (Cormack et al., 1996) results in a dramatically improved maturation of fluorescence at 37°C. Although this mutation is present in most of the popular varieties of FPs derived from *A. victoria*, mutations of additional residues have since been found to improve protein folding and the efficiency of chromophore formation (Tsien, 1998). Other important mutations include S30R for a faster folding rate (Pedelacq et al., 2006), Q69M, which improves photostability and resistance to chloride and low pH (Griesbeck et al., 2001), S72A, which enhances folding and stability (Cubitt et al., 1998), S147P for faster maturation (Kimata et al., 1997), N149K for improved folding rate (Cubitt et al., 1998), V163A, which reduces hydrophobicity and enhances folding (Crameri et al., 1996), and I167T for reduced thermosensitivity (Heim et al., 1994).

In addition to improving the physical properties of the proteins, the introduction of silent mutations into the coding sequence can optimize protein expression in the host organism and, therefore, also improve the use of FPs (Yang et al., 1996). It is important to note that virtually all FPs are oligomeric (either dimeric or tetrameric) in their natural environment. For example, wild-type *A. victoria* GFP is part of a heterotetrameric complex with aequorin (Ward, 2006), whereas *Renilla* (sea pansy) FPs often exist as dimers; most coral and anemone FPs occur naturally as tetramers (Shaner et al., 2007). To prevent oligomerization, further mutations were employed – such as the A206K mutation that practically eliminates dimerization of jellyfish-derived FPs (Zacharias et al., 2002). Eliminating tetramers in coral FPs has proven far more difficult, but efforts have led to substantial progress in producing monomeric coral FPs (Rizzo et al., 2010).

However, even after nearly 20 years of research, there is still no perfect fluorescent protein. Each experimental situation must be considered individually to determine the best FP variant to use. For example, if an experiment uses a green ion indicator dye, a red FP would probably be a better choice over a green or yellow FP. Besides color, several other parameters must be considered when evaluating FPs for a specific use. These include brightness (the combination of light absorbance and fluorescence quantum yield), protein stability (some FPs turn over quickly or change color over time), photostability (i.e. how fast the probe photobleaches), pH and temperature stability, and their effectiveness in fusions (i.e. interference of the FP with proper trafficking of the target protein). Although some of these

parameters are important in certain experiments, it is unlikely that all of them are a major consideration for any given experiment. Detailed discussions of these parameters and their relative trade-offs can be found elsewhere (Campbell and Davidson, 2010; Davidson and Campbell, 2009; Rizzo et al., 2010).

The fluorescent protein color palette

Because fluorescence is intrinsically a color-resolved technique, the most important consideration in choosing a FP is its spectral profile, that is, the color of its fluorescence. A broad range of FP variants that span nearly the entire visible spectrum has been developed and optimized (see poster panel 'Excitation and emission spectral properties of the brightest FPs'). The poster highlights the spectral and imaging properties of a few widely used FPs from across the spectrum. The FPs were purified and their extinction coefficients, quantum yields and spectral properties measured as previously described (Patterson et al., 1997). To compare the brightness of various FPs, each normalized excitation spectrum was multiplied by its peak molar extinction coefficient, and then divided by the peak molar extinction coefficient of EGFP. Similarly, each normalized emission spectrum was multiplied by its molecular brightness (molar extinction coefficient × quantum yield) and then divided by the brightness of EGFP.

The choices for the current best-performing FPs in each color class are based on a number of crucial factors, including maturation efficiency, spectral properties, photostability, monomeric character, brightness, fidelity in fusions and potential efficiency as a Förster resonance energy transfer (FRET) donor or acceptor. There are several key mutations that are repeatedly used in different FPs to enhance function as assayed by these parameters (see 'Critical mutations' in the poster). On the basis of overall performance, we consider mTagBFP (Subach et al., 2009) and mTurquoise (Goedhart et al., 2010) the brightest and most photostable blue and cyan FPs, respectively. mEGFP was the first generally reliable FP yet, because of its combination of positive attributes, it remains the gold standard with which to compare the performance of all other FPs. In the yellow and orange spectral regions, mVenus (Nagai et al., 2002) and mKO2 (Sakaue-Sawano et al., 2008) are useful because they mature rapidly and are both bright monomeric variants, even though they lack the level of photostability exhibited by mEGFP. In the orange-red spectral region, mCherry (Shaner et al., 2004) is widely used for many applications, but has been reported to aggregate when expressed within some fusions (Katayama et al., 2008). mApple (Shaner et al., 2008) can be used as an effective substitute for mCherry in most proteins fusions (such as connexins, α-tubulin and focal adhesions). Use of mApple helps to reduce artifacts, but its emission is blue-shifted by ~18 nm, which increases its spectral overlap with the yellow and orange FP variants. In the red to far-red region, mKate2 (Shcherbo et al., 2009) is currently the best choice in terms of brightness, photostability and performance in fusion proteins. The important photophysical properties of the selected FPs are summarized in the poster panel 'Fluorescent protein properties'.

By using multi-color fluorescence microscopy, FPs are often used in combination to examine interactions between their fusion partners (see poster panel 'Multi-color imaging with fluorescent protein fusions'). In the 2-color image shown here, pig kidney epithelial cells (the LLC-PK1 cell line) express mApple fused to human histone H2B, and mEGFP fused to human α-tubulin. The 3-color image shows HeLa cells that express mVenus fused to the SV40 T-antigen nuclear targeting signal to stain the nucleus, whereas mTurquoise and mCherry are fused to peptides targeted to the Golgi complex and mitochondria, respectively. In the 4-color panel, rabbit kidney (RK-13) cells are shown that express mCherry (fused to pyruvate dehydrogenase), mEGFP (fused to Lifeact), mTurquoise (fused to peroxisomal membrane protein), and mTagBFP (fused to H2B) to visualize the mitochondria, filamentous actin, peroxisomes, and nucleus. Finally, the 5-color assay combines the expression of mTagBFP, mTurquoise, mEGFP, mKO2 and mKate2 to label the nucleus, peroxisomes, endoplasmic reticulum, focal adhesions and mitochondria, respectively.

Additional applications of fluorescent proteins

The majority of research that makes use of GFP and other FPs spanning the color palette employs fluorescence microscopy, but no overview of FPs would be complete without mentioning some of the numerous applications that have been revolutionized through the availability of specific FPs (for reviews, see Chudakov et al., 2010; Rizzo et al., 2010; Wiedenmann et al., 2009). The advent of photoactivatable and photoswitching FPs has allowed the precise measurement of molecular diffusion and tracking, and given rise to super-resolution imaging methods and to single molecule microscopy (Day and Davidson, 2009; Shaner et al., 2007). Furthermore, an array of biosensors has been developed that are designed to detect intracellular ions and second messengers, and to measure membrane potential, receptor activation and a host of other metabolic functions. These biosensors are typically based on either cyclic permutations or FRET. In addition to its use in biosensors, FP-based FRET is also used to assess protein–protein interactions (Piston and Kremers, 2007), similar to the related approach of bimolecular fluorescence complementation (BiFC) (Kerppola, 2008). Naturally, most of these approaches still rely on the intrinsic color, brightness and other parameters of the FPs used. Interestingly, FPs have also been adapted for the use in localized photochemistry that is unrelated to fluorescence. One example for this is the KillerRed protein (Bulina et al., 2006), which generates reactive oxygen species (ROS) upon excitation, and is used for localized ROS production and to kill specific cells.

Future outlook

The blue, green, and yellow FPs appear to have reached their potential, but improvements are likely to continue for orange, red and – especially – far-red FPs. Red and far-red FPs are particularly important for the use with mammalian cells, where both autofluorescence and absorption of light by tissues are greatly reduced in the red compared with the blue and green portions of the visible spectrum. Thus, red and far-red wavelength FPs are particularly useful for the examination of thick specimens and whole animals. In fact, the current focus of FP development is centered on the discovery and creation of improved red and far-red FPs that perform as well as EGFP. Progress towards this goal has been impressive, many red FPs have been introduced that are monomeric, bright and fast-maturing.

This Cell Science at a Glance article focuses solely on autocatalytic FPs that do not require an extrinsic co-factor; recently, however, the expression of an FP using an added co-factor has pushed the spectral range into the infra-red (Shu et al., 2006). Whereas this first example might not be of practical use for many applications, it does represent a reasonable strategy; particularly because bioluminescent luciferase proteins have proven to be useful in vivo – even in mice – despite the fact that luciferin must be added as a co-factor. Another emerging class of genetic label utilize specific recognition sequences to recruit organic fluorophores (such as fluorescein) to yield a hybrid fluorescent system (Yano and Matsuzaki, 2009). These can be based on specific peptide sequences – such as the tetracysteine system (Martin et al., 2005), or may depend on enzyme-mediated ligation – such as the SNAP-TAG (Keppler et al., 2003). A second area of anticipated future growth is in the use of dynamic FP-based measurements. For example, the large potential of FPs in biosensors has been realized only now, and the number of

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FP-containing biosensor constructs is growing rapidly. The use of available structural information has made it possible to develop probes with improved sensitivity, and further improvements are likely to be made. The success of these efforts suggests that almost any biological parameter will ultimately be discernable if an appropriate FP-based biosensor is used. Finally, as microscopes with spectral separation capabilities become more commonly available, the expectation is that newer varieties of FPs will supplement the existing color palette. In particular, the current trend to develop both synthetic fluorescent probes and FPs is likely to expand the range of fluorophores that emit in the far-red and near-infrared.

Deposited in PMC for release after 12 months.

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