# Introduction to Fluorescent Proteins

The discovery of green fluorescent protein in the early 1960s ultimately heralded a new era in cell biology by enabling investigators to apply molecular cloning methods, fusing the fluorophore moiety to a wide variety of protein and enzyme targets, in order to monitor cellular processes in living systems using optical microscopy and related methodology. When coupled to recent technical advances in widefield fluorescence and confocal microscopy, including ultrafast low light level digital cameras and multitracking laser control systems, the green fluorescent protein and its color-shifted genetic derivatives have demonstrated invaluable service in many thousands of live-cell imaging experiments.

Figure 1 - Fluorescent Protein Labels in Living Cells
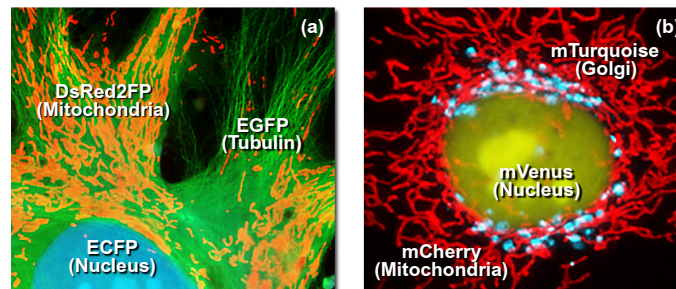
Osamu Shimomura and Frank Johnson, working at the Friday Harbor Laboratories of the University of Washington in 1961, first isolated a calcium-dependent bioluminescent protein from the *Aequorea victoria* jellyfish, which they named **aequorin**. During the isolation procedure, a second protein was observed that lacked the blue-emitting bioluminescent properties of aequorin, but was able to produce green fluorescence when illuminated with ultraviolet light. Due to this property, the protein was eventually christened with the unceremonious name of **green fluorescent protein** (**GFP**). Over the next two decades, researchers determined that aequorin and the green fluorescent protein work together in the light organs of the jellyfish to convert calcium-induced luminescent signals into the green fluorescence characteristic of the species.

Although the gene for green fluorescent protein was first cloned in 1992, the significant potential as a molecular probe was not realized until several years later when fusion products were used to track gene expression in bacteria and nematodes. Since these early studies, green fluorescent protein has been engineered to produce a vast number of variously colored mutants, fusion proteins, and biosensors that are broadly referred to as fluorescent proteins. More recently, fluorescent proteins from other species have been identified and isolated, resulting in further expansion of the color palette. With the rapid evolution of fluorescent protein technology, the utility of this genetically encoded fluorophore for a wide spectrum of

applications beyond the simple tracking of tagged biomolecules in living cells is now becoming fully appreciated.

Illustrated in Figure 1 are two examples of multiple fluorescent protein labeling in living cells using fusion products targeted at sub-cellular (organelle) locations. The opossum kidney cortex proximal tubule epithelial cell (**OK** line) presented in Figure 1(a) was transfected with a cocktail of fluorescent protein variants fused to peptide signals that mediate transport to either the nucleus (enhanced cyan fluorescent protein; **ECFP**), the mitochondria (DsRed fluorescent protein; **DsRed2FP**), or the microtubule network (enhanced green fluorescent protein; **EGFP**). A similar specimen consisting of human cervical adenocarcinoma epithelial cells (**HeLa** line) is depicted in Figure 1(b). The HeLa cells were co-transfected with sub-cellular localization vectors fused to cyan (**mTurquoise**) and yellow (**mVenus**) fluorescent protein coding sequences (Golgi complex and the nucleus, respectively), as well as the "Fruit" protein, mCherry, targeting the mitochondrial network.

Green fluorescent protein, and its mutated allelic forms, blue, cyan, and yellow fluorescent proteins are used to construct fluorescent chimeric proteins that can be expressed in living cells, tissues, and entire organisms, after transfection with the engineered vectors. Red fluorescent proteins have been isolated from other species, including coral reef organisms, and are similarly useful. The fluorescent protein technique avoids the problem of purifying, tagging, and introducing labeled proteins into cells or the task of producing specific antibodies for surface or internal antigens.

Properties and Modifications of Aequorea victoria Green Fluorescent Protein

Among the most important aspects of the green fluorescent protein to appreciate is that the entire 27 kiloDalton native peptide structure is essential to the development and maintenance of its fluorescence. It is remarkable that the principle fluorophore is derived from a triplet of adjacent amino acids: the serine, tyrosine, and glycine residues at locations 65, 66, and 67 (referred to as **Ser65**, **Tyr66**, and **Gly67**; see Figure 2). Although this simple amino acid motif is commonly found throughout nature, it does not generally result in fluorescence. What is unique to the fluorescent protein is that the location of this peptide triplet resides in the center of a remarkably stable barrel structure consisting of 11 *beta*-sheets folded into a tube.

Within the hydrophobic environment in the center of the green fluorescent protein, a reaction occurs between the carboxyl carbon of Ser65 and the amino nitrogen of Gly67 that results in the formation of an imidazolin-5-one heterocyclic nitrogen ring system (as illustrated in Figure 2). Further oxidation results in conjugation of the imidazoline ring with Tyr66 and maturation of a fluorescent species. It is important to note that the native green fluorescent protein fluorophore exists in two states. A protonated form, the predominant state, has an excitation maximum at 395 nanometers, and a less prevalent, unprotonated form that absorbs at approximately 475 nanometers. Regardless of the excitation wavelength, however, fluorescence emission has a maximum peak wavelength at 507 nanometers, although the peak is broad and not well defined.

Figure 2 - Green Fluorescent Protein Fluorophore Maturation
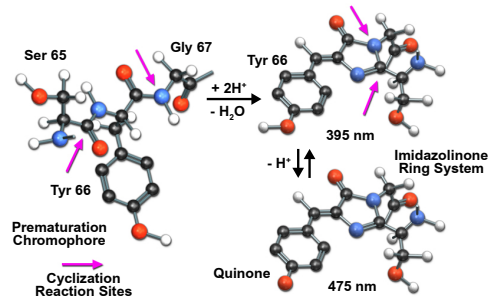
Two predominant features of the fluorescent protein fluorophore have important implications for its utility as a probe. First, the photophysical properties of green fluorescent protein as a fluorophore are quite complex and thus, the molecule can accommodate a considerable amount of modification. Many studies have focused on fine-tuning the fluorescence of native green fluorescent protein to provide a broad range of molecular probes, but the more significant and vast potential of employing the protein as a starting material for constructing advanced fluorophores cannot be understated. The second important feature of green fluorescent protein is that fluorescence is highly dependent on the molecular structure surrounding the tripeptide fluorophore.

Denaturation of green fluorescent protein destroys fluorescence, as might be expected, and mutations to residues surrounding the tripeptide fluorophore can dramatically alter the fluorescence properties. The packing of amino acid residues inside the *beta* barrel is extremely stable, which results in a very high fluorescence quantum yield (up to 80 percent). This tight protein structure also confers resistance to fluorescence variations due to fluctuations in pH, temperature, and denaturants such as urea. The high level of stability is generally altered in a negative manner by mutations in green fluorescent protein that perturb fluorescence, resulting in a reduction of quantum yield and greater environmental sensitivity. Although several of these defects can be overcome by additional mutations, derivative fluorescent proteins are generally more sensitive to the environment than the native species. These limitations should be seriously considered when designing experiments with genetic variants.

In order to adapt fluorescent proteins for use in mammalian systems, several basic modifications of the wild-type green fluorescent protein were undertaken and are now found in all commonly used variants. The first step was to optimize the maturation of fluorescence to a 37-degree Celsius environment. Maturation of the wild-type fluorophore is quite efficient at 28 degrees, but increasing the temperature to 37 degrees substantially reduces overall maturation and results in decreased fluorescence. Mutation of the phenylalanine residue at position 64 (**Phe64**) to leucine results in improved maturation of fluorescence at 37 degrees, which is at least equivalent to that observed at 28 degrees. This mutation is present in the most popular varieties of fluorescent proteins derived from *Aequorea victoria*, but is not the only mutation that improves folding at 37 degrees as other variants have been discovered.

In addition to improving the maturation at 37 degrees, the optimization of codon usage for mammalian expression has also improved overall brightness of green fluorescent protein expressed in mammalian cells. In all, over 190 silent mutations have been introduced into the coding sequence to enhance expression in human tissues. A Kozak translation initiation site (containing the nucleotide sequence **A/GCCAT**) was also introduced by insertion of valine as the second amino acid. These, along with a variety of other improvements (discussed below), have resulted in a very useful probe for live cell imaging of mammalian cells and are common to all of the currently used fluorescent probes derived from the original jellyfish protein.

The Fluorescent Protein Color Palette

A broad range of fluorescent protein genetic variants have been developed that feature fluorescence emission spectral profiles spanning almost the entire visible light spectrum (see Table 1). Mutagenesis efforts in the original *Aequorea victoria* jellyfish green fluorescent protein have resulted in new fluorescent probes that range in color from blue to yellow, and are some of the most widely used *in vivo* reporter molecules in biological research. Longer wavelength fluorescent proteins, emitting in the orange and red spectral regions, have been developed from the marine anemone, *Discosoma striata*, and reef corals belonging to the class **Anthozoa**. Still other species have been mined to produce similar proteins having cyan, green, yellow, orange, and deep red fluorescence emission. Developmental research efforts are ongoing to improve the brightness and stability of fluorescent proteins, thus improving their overall usefulness.

Table 1 - *Fluorescent Protein Properties*

Protein (Acronym)	Excitation Maximum (nm)	Emission Maximum (nm)	Molar Extinction Coefficient	Quantum Yield	*in vivo* Structure	Relative Brightness (% of EGFP)
GFP (wt)	395/475	509	21,000	0.77	Monomer*	48
Green Fluorescent Proteins						
EGFP	484	507	56,000	0.60	Monomer*	100
Emerald	487	509	57,500	0.68	Monomer*	116
Superfolder GFP	485	510	83,300	0.65	Monomer*	160
Azami Green	492	505	55,000	0.74	Monomer	121
mWasabi	493	509	70,000	0.80	Monomer	167
TagGFP	482	505	58,200	0.59	Monomer*	110
TurboGFP	482	502	70,000	0.53	Dimer	102
AcGFP	480	505	50,000	0.55	Monomer*	82
ZsGreen	493	505	43,000	0.91	Tetramer	117
T-Sapphire	399	511	44,000	0.60	Monomer*	79
Blue Fluorescent Proteins						
EBFP	383	445	29,000	0.31	Monomer*	27
EBFP2	383	448	32,000	0.56	Monomer*	53
Azurite	384	450	26,200	0.55	Monomer*	43
mTagBFP	399	456	52,000	0.63	Monomer	98
Cyan Fluorescent Proteins						

Protein (Acronym)	Excitation Maximum (nm)	Emission Maximum (nm)	Molar Extinction Coefficient	Quantum Yield	*in vivo* Structure	Relative Brightness (% of EGFP)
ECFP	439	476	32,500	0.40	Monomer*	39
mECFP	433	475	32,500	0.40	Monomer	39
Cerulean	433	475	43,000	0.62	Monomer*	79
mTurquoise	434	474	30,000	0.84	Monomer*	75
CyPet	435	477	35,000	0.51	Monomer*	53
AmCyan1	458	489	44,000	0.24	Tetramer	31
Midori-Ishi Cyan	472	495	27,300	0.90	Dimer	73
TagCFP	458	480	37,000	0.57	Monomer	63
mTFP1 (Teal)	462	492	64,000	0.85	Monomer	162
Yellow Fluorescent Proteins						
EYFP	514	527	83,400	0.61	Monomer*	151
Topaz	514	527	94,500	0.60	Monomer*	169
Venus	515	528	92,200	0.57	Monomer*	156
mCitrine	516	529	77,000	0.76	Monomer	174
YPet	517	530	104,000	0.77	Monomer*	238
TagYFP	508	524	64,000	0.60	Monomer	118
PhiYFP	525	537	124,000	0.39	Monomer*	144
ZsYellow1	529	539	20,200	0.42	Tetramer	25
mBanana	540	553	6,000	0.7	Monomer	13
Orange Fluorescent Proteins						
Kusabira Orange	548	559	51,600	0.60	Monomer	92
Kusabira Orange2	551	565	63,800	0.62	Monomer	118
mOrange	548	562	71,000	0.69	Monomer	146
mOrange2	549	565	58,000	0.60	Monomer	104
dTomato	554	581	69,000	0.69	Dimer	142
dTomato-Tandem	554	581	138,000	0.69	Monomer	283
TagRFP	555	584	100,000	0.48	Monomer	142

Protein (Acronym)	Excitation Maximum (nm)	Emission Maximum (nm)	Molar Extinction Coefficient	Quantum Yield	*in vivo* Structure	Relative Brightness (% of EGFP)
TagRFP-T	555	584	81,000	0.41	Monomer	99
DsRed	558	583	75,000	0.79	Tetramer	176
DsRed2	563	582	43,800	0.55	Tetramer	72
DsRed-Express (T1)	555	584	38,000	0.51	Tetramer	58
DsRed-Monomer	556	586	35,000	0.10	Monomer	10
mTangerine	568	585	38,000	0.30	Monomer	34
Red Fluorescent Proteins						
mRuby	558	605	112,000	0.35	Monomer	117
mApple	568	592	75,000	0.49	Monomer	109
mStrawberry	574	596	90,000	0.29	Monomer	78
AsRed2	576	592	56,200	0.05	Tetramer	8
mRFP1	584	607	50,000	0.25	Monomer	37
JRed	584	610	44,000	0.20	Dimer	26
mCherry	587	610	72,000	0.22	Monomer	47
HcRed1	588	618	20,000	0.015	Dimer	1
mRaspberry	598	625	86,000	0.15	Monomer	38
dKeima-Tandem	440	620	28,800	0.24	Monomer	21
HcRed-Tandem	590	637	160,000	0.04	Monomer	19
mPlum	590	649	41,000	0.10	Monomer	12
AQ143	595	655	90,000	0.04	Tetramer	11
* Weak Dimer						

Presented in Table 1 is a compilation of properties displayed by several of the most popular and useful fluorescent protein variants. Along with the common name and/or acronym for each fluorescent protein, the peak absorption and emission wavelengths (given in nanometers), molar extinction coefficient, quantum yield, relative brightness, and *in vivo*structural associations are listed. The computed brightness values were derived from the product of the molar extinction coefficient and quantum yield, divided by the value for EGFP. This listing was created from scientific and commercial literature resources and is not intended to be comprehensive, but instead represents fluorescent protein derivatives that

have received considerable attention in the literature and may prove valuable in research efforts. Furthermore, the absorption and fluorescence emission spectra listed in tables and illustrated below were recorded under controlled conditions and are normalized for comparison and display purposes only. In actual fluorescence microscopy investigations, spectral profiles and wavelength maxima may vary due to environmental effects, such as pH, ionic concentration, and solvent polarity, as well as fluctuations in localized probe concentration. Therefore, the listed extinction coefficients and quantum yields may differ from those actually observed under experimental conditions.

Green Fluorescent Proteins

Although native green fluorescent protein produces significant fluorescence and is extremely stable, the excitation maximum is close to the ultraviolet range. Because ultraviolet light requires special optical considerations and can damage living cells, it is generally not well suited for live cell imaging with optical microscopy. Fortunately, the excitation maximum of green fluorescent protein is readily shifted to 488 nanometers (in the cyan region) by introducing a single point mutation altering the serine at position 65 into a threonine residue (**S65T**). This mutation is featured in the most popular variant of green fluorescent protein, termed **enhanced** GFP (**EGFP**), which is commercially available in a wide range of vectors offered by BD Biosciences Clontech, one of the leaders in fluorescent protein technology. Furthermore, the enhanced version can be imaged using commonly available filter sets designed for fluorescein and is among the brightest of the currently available fluorescent proteins. These features have rendered enhanced green fluorescent protein one of the most popular probes and the best choice for most single-label fluorescent protein experiments. The only drawbacks to the use of EGFP are a slight sensitivity to pH and a weak tendency to dimerize.

In addition to enhanced green fluorescent protein, several other variants are currently being used in live-cell imaging. One of the best of these in terms of photostability and brightness may be the **Emerald** variant, but lack of a commercial source has limited its use. Several sources provide humanized green fluorescent protein variants that offer distinct advantages for fluorescence resonance energy transfer (**FRET**) experiments. Substitution of the phenylalanine residue at position 64 for leucine (**F64L**; **GFP2**) yields a mutant that retains the 400-nanometer excitation peak and can be coupled as an effective partner for enhanced yellow fluorescent protein. A variant of the **S65C** mutation (normally substituting cysteine for serine) having a peak excitation at 474 nanometers has been introduced commercially as a more suitable FRET partner for enhanced blue fluorescent protein than the red-shifted enhanced green version. Finally, a reef coral protein, termed **ZsGreen1** and having an emission peak at 505 nanometers, has been introduced as a substitute for enhanced green fluorescent protein. When expressed in mammalian cells, ZsGreen1 is very bright relative to EGFP, but has limited utility in producing fusion mutants and, similar to other reef coral proteins, has a tendency to form tetramers.

Yellow Fluorescent Proteins

The family of yellow fluorescent proteins was initiated after the crystal structure of green fluorescent protein revealed that threonine residue 203 (**Thr203**) was near the chromophore. Mutation of this residue to tyrosine was introduced to stabilize the excited state dipole moment of the chromophore and resulted in a 20-nanometer shift to longer wavelengths for both the excitation and emission spectra. Further refinements led to the development of the **enhanced** yellow fluorescent protein (**EYFP**), which is one of the brightest and most widely used fluorescent proteins. The brightness and fluorescence emission spectrum of enhanced yellow fluorescent protein combine to make this probe an excellent candidate for multicolor imaging experiments in fluorescence microscopy. Enhanced yellow fluorescent protein is also useful for energy transfer experiments when paired with enhanced cyan fluorescent protein

(ECFP) or GFP2. However, yellow fluorescent protein presents some problems in that it is very sensitive to acidic pH and loses approximately 50 percent of its fluorescence at pH 6.5. In addition, EYFP has also been demonstrated to be sensitive to chloride ions and photobleaches much more readily than the green fluorescent proteins.

Figure 3 - Spectral Profiles of Common Fluorescent Proteins
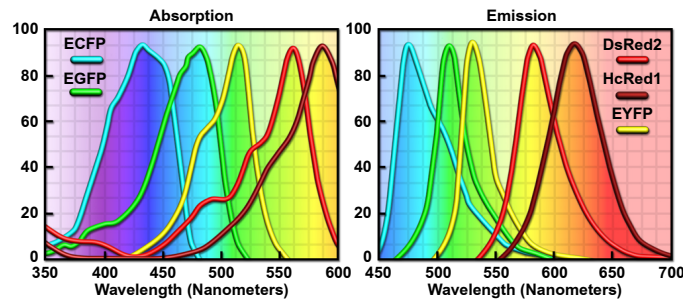
Continued development of fluorescent protein architecture for yellow emission has solved several of the problems with the yellow probes. The Citrine variant of yellow fluorescent protein is very bright relative to EYFP and has been demonstrated to be much more resistant to photobleaching, acidic pH, and other environmental effects. Another derivative, named Venus, is the fastest maturing and one of the brightest yellow variants developed to date. The coral reef protein, ZsYellow1, originally cloned from a *Zoanthus* species native to the Indian and Pacific oceans, produces true yellow emission and is ideal for multicolor applications. Like ZsGreen1, this derivative is not as useful for creating fusions as EYFP and has a tendency to form tetramers. Many of the more robust yellow fluorescent protein variants have been important for quantitative results in FRET studies and are potentially useful for other investigations as well.

Illustrated in Figure 3 are the absorption and emission spectral profiles for many of the commonly used and commercially available fluorescent proteins, which span the visible spectrum from cyan to far red. The variants derived from *Aequorea victoria* jellyfish, including enhanced cyan, green, and yellow fluorescent proteins, exhibit peak emission wavelengths ranging from 425 to 525 nanometers. Fluorescent proteins derived from coral reefs, DsRed2 and HcRed1 (discussed below), emit longer wavelengths but suffer from oligomerization artifacts in mammalian cells.

Blue and Cyan Fluorescent Proteins

The blue and cyan variants of green fluorescent protein resulted from direct modification of the tyrosine residue at position 66 (Tyr66) in the native fluorophore (see Figure 2). Conversion of this amino acid to histidine results in blue emission having a wavelength maxima at 450 nanometers, whereas conversion to tryptamine results in a major fluorescence peak around 480 nanometers along with a shoulder that peaks around 500 nanometers. Both probes are only weakly fluorescent and require secondary mutations to increase folding efficiency and overall brightness. Even with modifications, the enhanced versions in this class of fluorescent protein (EBFP and ECFP) are only about 25 to 40 percent as bright as enhanced green fluorescent protein. In addition, excitation of blue and cyan fluorescent proteins is most efficient in spectral regions that are not commonly used, so specialized filter sets and laser sources are required.

Despite the drawbacks of blue and cyan fluorescent proteins, the widespread interest in multicolor labeling and FRET has popularized their application in a number of investigations. This is especially true for enhanced cyan fluorescent protein, which can be excited off-peak by an argon-ion laser (using the 457-nanometer spectral line) and is significantly more resistant to photobleaching than the blue derivative. In contrast to other fluorescent proteins, there has not been a high level of interest for designing better probes in the blue region of the visible light spectrum, and a majority of the developmental research on fluorophores in this class has been focused on cyan variants.

Figure 4 - Spectral Profiles of Fluorescent Protein FRET Pail Variants
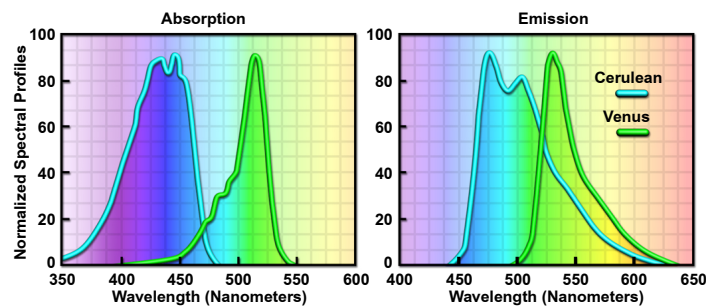
Among the improved cyan fluorescent proteins that have been introduced, **AmCyan1** and an enhanced cyan variant termed **Cerulean** show the most promise. Derived from the reef coral, *Anemonia majano*, the AmCyan1 fluorescent protein variant has been optimized with human codons to generate a high relative brightness level and resistance to photobleaching when compared to enhanced cyan fluorescent protein during mammalian expression. On the downside, similar to most of the other reef coral proteins, this probe has a tendency to form tetramers. The Cerulean fluorescent probe was developed by site-directed mutagenesis of enhanced cyan fluorescent protein to yield a higher extinction coefficient and improved quantum yield. Cerulean is at least 2-fold brighter than enhanced cyan fluorescent protein and has been demonstrated to significantly increase the signal-to-noise ratio when coupled with yellow-emitting fluorescent proteins, such as Venus (see **Figure 4**), in FRET investigations.
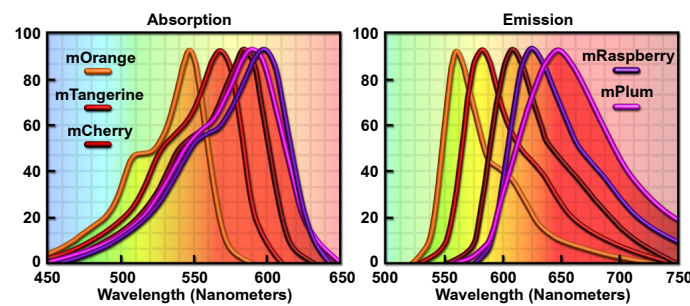
Red Fluorescent Proteins

A major goal of fluorescent protein development has become the construction of a red-emitting derivative that equals or exceeds the advanced properties of enhanced green fluorescent protein. Among the advantages of a suitable red fluorescent protein are the potential compatibility with existing confocal and widefield microscopes (and their filter sets), along with an increased capacity to image entire animals, which are significantly more transparent to red light. Because the construction of red-shifted mutants from the *Aequorea victoria* jellyfish green fluorescent protein beyond the yellow spectral region has proven largely unsuccessful, investigators have turned their search to the tropical reef corals.

The first coral-derived fluorescent protein to be extensively utilized was derived from *Discosoma striata* and is commonly referred to as **DsRed**. Once fully matured, the fluorescence emission spectrum of DsRed features a peak at 583 nanometers whereas the excitation spectrum has a major peak at 558 nanometers and a minor peak around 500 nanometers. Several problems are associated with using DsRed, however. Maturation of DsRed fluorescence occurs slowly and proceeds through a time period when fluorescence emission is in the green region. Termed the **green state**, this artifact has proven problematic for multiple labeling experiments with other green fluorescent proteins because of the spectral

overlap. Furthermore, DsRed is an obligate tetramer and can form large protein aggregates in living cells. Although these features are inconsequential for the use of DsRed as a reporter of gene expression, the usefulness of DsRed as an epitope tag is severely limited. In contrast to the jellyfish fluorescent proteins, which have been successfully used to tag hundreds of proteins, DsRed conjugates have proven much less successful and are often toxic.

A few of the problems with DsRed fluorescent proteins have been overcome through mutagenesis. The second-generation DsRed, known as **DsRed2**, contains several mutations at the peptide amino terminus that prevent formation of protein aggregates and reduce toxicity. In addition, the fluorophore maturation time is reduced with these modifications. The DsRed2 protein still forms a tetramer, but it is more compatible with green fluorescent proteins in multiple labeling experiments due to the quicker maturation. Further reductions in maturation time have been realized with the third generation of DsRed mutants, which also display an increased brightness level in terms of peak cellular fluorescence. Red fluorescence emission from **DsRed-Express** can be observed within an hour after expression, as compared to approximately six hours for DsRed2 and 11 hours for DsRed. A yeast-optimized variant, termed **RedStar**, has been developed that also has an improved maturation rate and increased brightness. The presence of a green state in DsRed-Express and RedStar is not apparent, rendering these fluorescent proteins the best choice in the orange-red spectral region for multiple labeling experiments. Because these probes remain obligate tetramers, they are not the best choice for labeling proteins.

Figure 5 - Spectral Profiles of Orange and Red Monometric Fluorescent Proteins

Several additional red fluorescent proteins showing a considerable amount of promise have been isolated from the reef coral organisms. One of the first to be adapted for mammalian applications is **HcRed1**, which was isolated from *Heteractis crispa* and is now commercially available. HcRed1 was originally derived from a non-fluorescent chromoprotein that absorbs red light through mutagenesis to produce a weakly fluorescent obligate dimer having an absorption maximum at 588 nanometers and an emission maximum of 618 nanometers. Although the fluorescence emission spectrum of this protein is adequate for separation from DsRed, it tends to co-aggregate with DsRed and is far less bright. An interesting HcRed construct containing two molecules in tandem has been produced to overcome dimerization that, in principle, occurs preferentially within the tandem pairing to produce a monomeric tag. However, because the overall brightness of this twin protein has not yet been improved, it is not a good choice for routine applications in live-cell microscopy.

Developing Monomeric Fluorescent Protein Variants

In their natural states, most fluorescent proteins exist as dimers, tetramers, or higher order oligomers. Likewise, *Aequorea victoria* green fluorescent protein is thought to participate in a tetrameric complex with aequorin, but this phenomenon has only been observed at very high

protein concentrations and the tendency of jellyfish fluorescent proteins to dimerize is generally very weak (having a dissociation constant greater than 100 micromolar). Dimerization of fluorescent proteins has thus not generally been observed when they are expressed in mammalian systems. However, when fluorescent proteins are targeted to specific cellular compartments, such as the plasma membrane, the localized protein concentration can theoretically become high enough to permit dimerization. This is a particular concern when conducting FRET experiments, which can yield complex data sets that are easily compromised by dimerization artifacts.

The construction of monomeric DsRed variants has proven to be a difficult task. More than 30 amino acid alterations to the structure were required for the creation of the first-generation monomeric DsRed protein (termed **RFP1**). However, this derivative exhibits significantly reduced fluorescence emission compared to the native protein and photobleaches very quickly, rendering it much less useful then monomeric green and yellow fluorescent proteins. Mutagenesis research efforts, including novel techniques such as somatic hypermutation, are continuing in the search for yellow, orange, red, and deep red fluorescent protein variants that further reduce the tendency of these potentially efficacious biological probes to self-associate while simultaneously pushing emission maxima towards longer wavelengths.

Improved monomeric fluorescent proteins are being developed that have increased extinction coefficients, quantum yields, and photostability, although no single variant has yet been optimized by all criteria. In addition, the expression problems with obligate tetrameric red fluorescent proteins are being overcome by the efforts to generate monomeric variants, which have yielded derivatives that are more compatible with biological function.

Perhaps the most spectacular development on this front has been the introduction of a new harvest of fluorescent proteins derived from monomeric red fluorescent protein through directed mutagenesis targeting the **Q66** and **Y67** residues. Named for fruits that reflect colors similar to the fluorescence emission spectral profile (see Table 1 and Figure 5), this cadre of monomeric fluorescent proteins exhibits maxima at wavelengths ranging from 560 to 610 nanometers. Further extension of this class through iterative somatic hypermutation yielded fluorescent proteins with emission wavelengths up to 650 nanometers. These new proteins essentially fill the gap between the most red-shifted jellyfish fluorescent proteins (such as Venus), and the coral reef red fluorescent proteins. Although several of these new fluorescent proteins lack the brightness and stability necessary for many imaging experiments, their existence is encouraging as it suggests the eventuality of bright, stable, monomeric fluorescent proteins across the entire visible spectrum.

Optical Highlighters

One of the most interesting developments in fluorescent protein research has been the application of these probes as **molecular** or **optical highlighters** (see Table 2), which change color or emission intensity as the result of external photon stimulation or the passage of time. As an example, a single point mutation to the native jellyfish peptide creates a photoactivatable version of green fluorescent protein (known as **PA-GFP**) that enables photoconversion of the excitation peak from ultraviolet to blue by illumination with light in the 400-nanometer range. Unconverted PA-GFP has an excitation peak similar in profile to that of the wild type protein (approximately 395 to 400 nanometers). After photoconversion, the excitation peak at 488 nanometers increases approximately 100-fold. This event evokes very high contrast differences between the unconverted and converted pools of PA-GFP and is useful for tracking the dynamics of molecular subpopulations within a cell. Illustrated in Figure 6(a) is a transfected living mammalian cell containing PA-GFP in the cytoplasm being imaged with 488-nanometer argon-ion laser excitation before (Figure 6(a)) and after (Figure 6(d)) photoconversion with a 405-nanometer blue diode laser.

Table 2 - Properties of Selected Optical Highlighters

| Protein (Acronym)	Excitation Maximum (nm)	Emission Maximum (nm)	Molar Extinction Coefficient	Quantum Yield	*in vivo* Structure	Relative Brightness (% of EGFP)
PA-GFP (G)	504	517	17,400	0.79	Monomer	41
PS-CFP (C)	402	468	34,000	0.16	Monomer	16
PS-CFP (G)	490	511	27,000	0.19	Monomer	15
PA-mRFP1 (R)	578	605	10,000	0.08	Monomer	3
CoralHue Kaede (G)	508	518	98,800	0.88	Tetramer	259
CoralHue Kaede (R)	572	580	60,400	0.33	Tetramer	59
wtKikGR (G)	507	517	53,700	0.70	Tetramer	112
wtKikGR (R)	583	593	35,100	0.65	Tetramer	68
mKikGR (G)	505	515	49,000	0.69	Monomer	101
mKikGR (R)	580	591	28,000	0.63	Monomer	53
dEosFP-Tandem (G)	506	516	84,000	0.66	Monomer	165
dEosFP-Tandem (R)	569	581	33,000	0.60	Monomer	59
mEos2FP (G)	506	519	56,000	0.84	Monomer	140
mEos2FP (R)	573	584	46,000	0.66	Monomer	90
Dendra2 (G)	490	507	45,000	0.50	Monomer	67
Dendra2 (R)	553	573	35,000	0.55	Monomer	57
CoralHue Dronpa (G)	503	518	95,000	0.85	Monomer	240
Kindling (KFP1)	580	600	59,000	0.07	Tetramer	12

Other fluorescent proteins can also be employed as optical highlighters. Three-photon excitation (at less than 760 nanometers) of DsRed fluorescent protein is capable of converting the normally red fluorescence to green. This effect is likely due to selective photobleaching of the red chromophores in DsRed, resulting in observable fluorescence from the green state. The **Timer** variant of DsRed gradually turns from bright green (500-nanometer emission) to bright red (580-nanometer emission) over the course of several hours. The relative ratio of green to red fluorescence can then be used to gather temporal data for gene expression investigations.

A photoswitchable optical highlighter, termed **PS-CFP**, derived by mutagenesis of a green fluorescent protein variant, has been observed to transition from cyan to green fluorescence

upon illumination at 405 nanometers (note photoconversion of the central cell in **Figures 6(b) and 6(e)**). Expressed as a monomer, this probe is potentially useful in photobleaching, photoconversion and photoactivation investigations. However, the fluorescence from PS-CFP is approximately 2.5-fold dimmer than PA-GFP and is inferior to other highlighters in terms of photoconversion efficiency (the 40-nanometer shift in fluorescence emission upon photoconversion is less than observed with similar probes). Additional mutagenesis of this or related fluorescent proteins has the potential to yield more useful variants in this wavelength region.

Figure 6 - Optical Highlighter Fluorescent Proteins
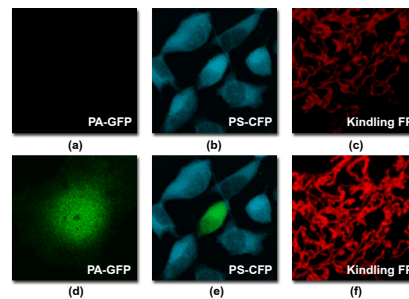
Optical highlighters have also been developed in fluorescent proteins cloned from coral and anemone species. **Kaede**, a fluorescent protein isolated from stony coral, photoconverts from green to red in the presence of ultraviolet light. Unlike PA-GFP, the conversion of fluorescence in Kaede occurs by absorption of light that is spectrally distinct from its illumination. Unfortunately, this protein is an obligate tetramer, making it less suitable fur use as an epitope tag than PA-GFP. Another tetrameric stony coral (*Lobophyllia hemprichii*) fluorescent protein variant, termed **EosFP** (see Table 2), emits bright green fluorescence that changes to orange-red when illuminated with ultraviolet light at approximately 390 nanometers. In this case, the spectral shift is produced by a photo-induced modification involving a break in the peptide backbone adjacent to the chromophore. Further mutagenesis of the "wild type" EosFP protein yielded monomeric derivatives, which may be useful in constructing fusion proteins.

A third non-*Aequorea* optical highlighter, the **Kindling** fluorescent protein (**KFP1**) has been developed from a non-fluorescent chromoprotein isolated in *Anemonia sulcata*, and is now commercially available (Evrogen). Kindling fluorescent protein does not exhibit emission until illuminated with green light. Low-intensity light results in a transient red fluorescence that decays over a few minutes (see the mitochondria in **Figure 6(c)**). Illumination with blue light quenches the kindled fluorescence immediately, allowing tight control over fluorescent labeling. In contrast, high-intensity illumination results in irreversible kindling and allows for stable highlighting similar to PA-GFP (**Figure 6(f)**). The ability to precisely control fluorescence is particular useful when tracking particle movement in a crowded environment. For example, this approach has been successfully used to track the fate of neural plate cells in developing *Xenopus* embryos and the movement of individual mitochondria in PC12 cells.

As the development of optical highlighters continues, fluorescent proteins useful for optical marking should evolve towards brighter, monomeric variants that can be easily photoconverted and display a wide spectrum of emission colors. Coupled with these advances, microscopes equipped to smoothly orchestrate between illumination modes for fluorescence observation and regional marking will become commonplace in cell biology laboratories. Ultimately, these innovations have the potential to make significant achievements in the spatial and temporal dynamics of signal transduction systems.

Fluorescent Protein Vectors and Gene Transfer

Fluorescent proteins are quite versatile and have been successfully employed in almost every biological discipline from microbiology to systems physiology. These ubiquitous probes have been extremely useful as reporters for gene expression studies in cultured cells and tissues, as well as living animals. In live cells, fluorescent proteins are most commonly employed to track the localization and dynamics of proteins, organelles, and other cellular compartments. A variety of techniques have been developed to construct fluorescent protein fusion products and enhance their expression in mammalian and other systems. The primary vehicles for introducing fluorescent protein chimeric gene sequences into cells are genetically engineered bacterial plasmids and viral vectors.

Fluorescent protein gene fusion products can be introduced into mammalian and other cells using the appropriate vector (usually a plasmid or virus) either transiently or stably. In transient, or temporary, gene transfer experiments (often referred to as **transient transfection**), plasmid or viral DNA introduced into the host organism does not necessarily integrate into the chromosomes, but can be expressed in the cytoplasm for a short period of time. Expression of gene fusion products, easily monitored by the observation of fluorescence emission using a filter set compatible with the fluorescent protein, usually takes place over a period of several hours after transfection and continues for 72 to 96 hours after introduction of plasmid DNA into mammalian cells. In many cases, the plasmid DNA can be incorporated into the genome in a permanent state to form stably transformed cell lines. The choice of transient or stable transfection depends upon the target objectives of the investigation.

Figure 7 - EYFP Endoplasmic Reticulum Localization Vector
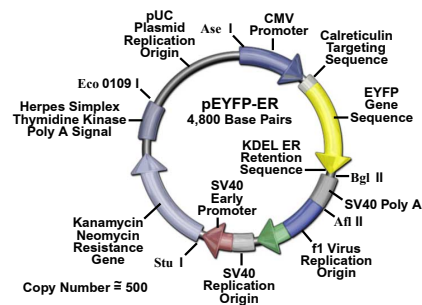
The basic plasmid vector configuration useful in fluorescent protein gene transfer experiments has several requisite components. The plasmid must contain prokaryotic nucleotide sequences coding for a bacterial replication origin for DNA and an antibiotic resistance gene. These elements, often termed **shuttle** sequences, allow propagation and selection of the plasmid within a bacterial host to generate sufficient quantities of the vector for mammalian transfections. In addition, the plasmid must contain one or more eukaryotic genetic elements that control the initiation of messenger RNA transcription, a mammalian polyadenylation signal, an intron (optional), and a gene for co-selection in mammalian cells. Transcription elements are necessary for the mammalian host to express the gene fusion product of interest, and the selection gene is usually an antibiotic that bestows resistance to cells containing the plasmid. These general features vary according to plasmid design, and many vectors have a wide spectrum of additional components suited for particular applications.

Illustrated in Figure 7 is the restriction enzyme and genetic map of a commercially available (BD Biosciences Clontech) bacterial plasmid derivative containing the coding sequence for

enhanced yellow fluorescent protein fused to the endoplasmic reticulum targeting sequence of calreticulin (a resident protein). Expression of this gene product in susceptible mammalian cells yields a chimeric peptide containing EYFP localized to the endoplasmic reticulum membrane network, designed specifically for fluorescent labeling of this organelle. The host vector is a derivative of the **pUC** high copy number (approximately 500) plasmid containing the bacterial replication origin, which makes it suitable for reproduction in specialized *E. coli* strains. The kanamycin antibiotic gene is readily expressed in bacteria and confers resistance to serve as a selectable marker.

Additional features of the EYFP vector presented above are a human cytomegalovirus (**CMV**) promoter to drive gene expression in transfected human and other mammalian cell lines, and an **f1** bacteriophage replication origin for single-stranded DNA production. The vector backbone also contains a simian virus 40 (**SV40**) replication origin, which is active in mammalian cells that express the SV40 T-antigen. Selection of stable transfectants with the antibiotic **G418** is enabled with a neomycin resistance cassette consisting of the SV40 early promoter, the neomycin resistance gene (aminoglycoside 3'-phosphotransferase), and polyadenylation signals from the herpes simplex virus thymidine kinase (**HSV-TK**) for messenger stability. Six unique restriction enzyme sites (see Figure 7) are present on the plasmid backbone, which increases the versatility of this plasmid.

Propagation, Isolation, and Transfection of Fluorescent Protein Plasmids

Successful mammalian transfection experiments rely on the use of high quality plasmid or viral DNA vectors that are relatively free of bacterial endotoxins. In the native state, circular plasmid DNA molecules exhibit a tertiary **supercoiled** conformation that twists the double helix around itself several times. For many years, the method of choice for supercoiled plasmid and virus DNA purification was cesium chloride density gradient centrifugation in the presence of an intercalation agent (such as ethidium bromide or propidium iodide). This technique, which is expensive in terms of both equipment and materials, segregates the supercoiled (plasmid) DNA from linear chromosomal and nicked circular DNA according to buoyant density, enabling the collection of high purity plasmid DNA. Recently, simplified ion-exchange column chromatography methods (commonly termed a **mini-prep**) have largely supplanted the cumbersome and time-consuming centrifugation protocol to yield large quantities of endotoxin-free plasmid DNA in a relatively short period of time.

Specialized bacterial mutants, termed **competent** cells, have been developed for convenient and relatively cheap amplification of plasmid vectors. The bacteria contain a palette of mutations that render them particularly susceptible to plasmid replication, and have been chemically permeabilized for transfer of the DNA across the membrane and cell wall in a procedure known as **transformation**. After transformation, the bacteria are grown to logarithmic phase in the presence of the selection antibiotic dictated by the plasmid. The bacterial culture is concentrated by centrifugation and disrupted by lysis with an alkaline detergent solution containing enzymes to degrade contaminating RNA. The lysate is then filtered and placed on the ion-exchange column. Unwanted materials, including RNA, DNA, and proteins, are thoroughly washed from the column before the plasmid DNA is eluted using a high salt buffer. Alcohol (isopropanol) precipitation concentrates the eluted plasmid DNA, which is collected by centrifugation, washed, and redissolved in buffer. The purified plasmid DNA is ready for duty in transfection experiments.

Figure 8 - Lipid-Mediated Transfection in Mammalian Cells
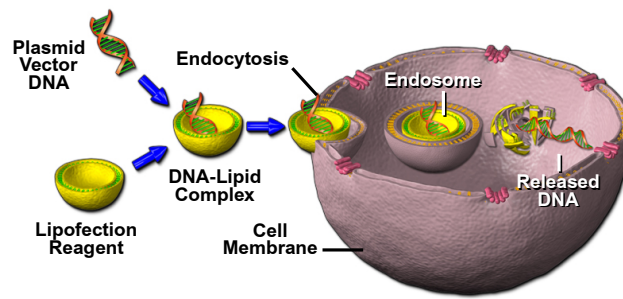
Mammalian cells used for transfection must be in excellent physiological condition and growing in logarithmic phase during the procedure. A wide spectrum of transfection reagents has been commercially developed to optimize uptake of plasmid DNA by cultured cells. These techniques range from simple calcium phosphate precipitation to sequestering the plasmid DNA in lipid vesicles that fuse to the cell membrane and deliver the contents to the cytoplasm (as illustrated in **Figure 8**). Collectively termed **lipofection**, the lipid-based technology has met with widespread acceptance due to its effectiveness in a large number of popular cell lines, and it is now the method of choice for most transfection experiments.

Although transient transfections usually result in the loss of plasmid gene product over a relatively short period of time (several days), stably transfected cell lines continue to produce the guest proteins on a continuous long-term basis (ranging from months to years). Stable cell lines can be selected using antibiotic markers present in the plasmid backbone (see **Figure 7**). One of the most popular antibiotics for selection of stable transfectants in mammalian cell lines is the protein synthesis-inhibiting drug G418, but the required dose varies widely according to each cell line. Other common antibiotics, including **hydromycin-B** and **puromycin**, have also been developed for stable cell selection, as have genetic markers. The most efficient method of obtaining stable cell lines is to employ a high efficiency technique for the initial transfection. In this regard, **electroporation** has proven to generate stable transfectants with linearized plasmids and purified genes. Electroporation applies short, high voltage pulses to a cellular suspension to induce pore formation in the plasma membrane, subsequently allowing the transfection DNA to enter the cell. Specialized equipment is necessary for electroporation, however, the technique is comparable in expense to lipofection reagents when a large number of transfections are performed.

The Future of Fluorescent Proteins

The focus of current fluorescent protein development is centered on two basic goals. The first is to perfect and fine-tune the current palette of blue to yellow fluorescent proteins derived from *Aequorea victoria* jellyfish, while the second aim is to develop monomeric fluorescent proteins emitting in the orange to far red regions of the visible light spectrum. Progress toward these goals has been quite impressive, and it is not inconceivable that near-infrared fluorescent proteins loom on the horizon.

The latest generation of jellyfish variants has solved most of the deficiencies of the first generation fluorescent proteins, particularly for the yellow and green derivatives. The search for a monomeric, bright, and fast-maturing red fluorescent protein has resulted in several new and interesting classes of fluorescent proteins, particularly those derived from coral species. Development of existing fluorescent proteins, together with new technologies, such as insertion of unnatural amino acids, will further expand the color palette. As optical spectral separation techniques become better developed and more widespread, these new varieties will supplement the existing palette, especially in the yellow and red regions of the spectrum.

The current trend in fluorescent probe technology is to expand the role of dyes that fluoresce into the far red and near infrared. In mammalian cells, both autofluorescence and the absorption of light are greatly reduced at the red end of the spectrum. Thus, the development of far red fluorescent probes would be extremely useful for the examination of thick specimens and entire animals. Given the success of fluorescent proteins as reporters in transgenic systems, the use of far red fluorescent proteins in whole organisms will become increasingly important in the coming years.

Finally, the tremendous potential in fluorescent protein applications for the engineering of biosensors is just now being realized. The number of biosensor constructs is rapidly growing. By using structural information, development of these probes has led to improved sensitivity and will continue to do so. The success of these endeavors certainly suggests that almost any biological parameter will be measurable using the appropriate fluorescent protein-based biosensor.

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