Proteins that glow in green and blue

An intrinsically fluorescent protein from a Pacific jellyfish promises to become an important power tool in experimental biology. Mutant forms of this green fluorescent protein with altered spectral characteristics have recently been constructed. It is now possible to envision a range of derivatives optimized for specific applications.

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Upon agitation, the jellyfish *Aequorea victoria* emits green light from between its tentacles. This green light is the product of two closely-associated proteins: a calcium-activated luciferase known as aequorin, and green fluorescent protein (GFP). Aequorin generates blue light; GFP alters the wavelength of this light from blue (λ_{max} 470 nm) to green (λ_{max} 509 nm) [1]. The transfer of energy between the two proteins is thought to take place by a non-radiative mechanism [2]. Remarkably, GFP converts the blue light to green without the need for cofactors or exogenous substrates; the fluorophore consists of an apparently unprecedented cyclic tripeptide within the protein that appears to be formed by a novel autocatalytic mechanism [3,4]. GFP has now been expressed in organisms ranging from *Escherichtia coli* to mammalian cells, and has been found to be strongly fluorescent in every cell type tested.

Wild-type GFP is a monomeric protein with a molecular weight of 27 kDa. It has an excitation maximum of 395 nm with a secondary maximum at 475 nm; light emission peaks at 509 nm [1]. The fluorophore is unique in that it is derived by cyclization of a tripeptide and subsequent oxidation of the cyclized tyrosyl side chain [5]; a reaction mechanism proposed by Heim *et al.* [5] is shown in Fig. 1. The catalyst for this unprecedented cyclization reaction is unknown, but oxidation of the tyrosyl side chain occurs spontaneously in the presence of air, with a time constant of ~2 h in solution [5,6]. The local chemical environment within the protein amplifies and red-shifts both emission and excitation maxima of the fluorophore; a proteolytic peptide that contains the fluorophore shows only weak fluorescence at a shorter wavelength (λ_{max} 430 nm) [4].

Applications of GFP in experimental biology

As the first intrinsically-fluorescent protein, GFP holds tremendous promise as a tool in experimental biology. At the longer wavelength excitation maximum (475 nm), wild-type GFP is resistant to photobleaching and is easily visualized with the fluorescein filter set fitted to nearly all fluorescence microscopes (mutant forms of GFP with even better spectral properties have been identified very recently; see below). GFP's potential as a reporter molecule for gene-expression studies was strikingly demonstrated by Chalfie *et al.* [7], who transformed *E. coli* with a GFP expression construct. The bacterial colonies fluoresced brightly when illuminated with a long-wave UV source. Chalfie *et al.* [7] also observed green fluorescent neurons in transgenic *Caenorhabditis elegans* that expressed the GFP

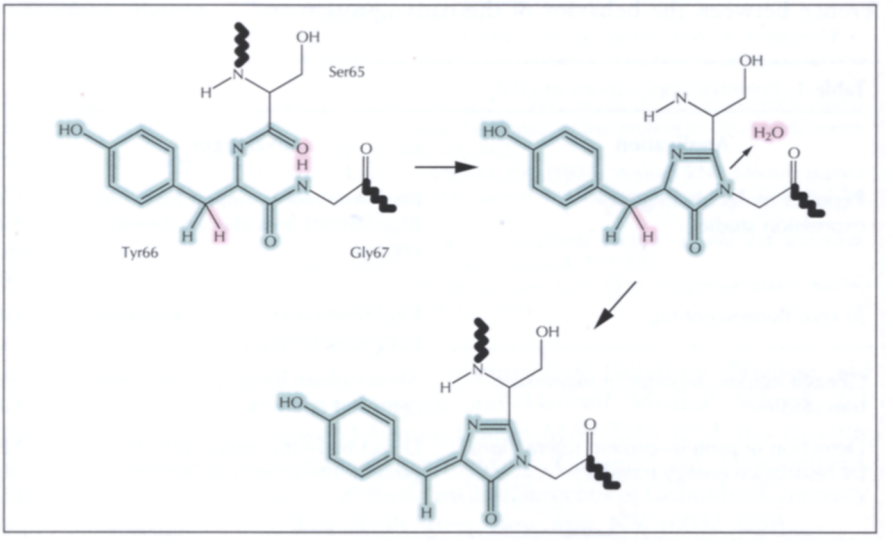

Fig.1. Proposed mechanism of fluorophore formation in GFP [5]. The peptide carbonyl of Ser65 and the peptide amide of Gly67 undergo a condensation reaction to form the imidazolidin-5-one; the catalyst for this step has not been identified. Oxidation of the Tyr66 side chain completes the fluorophore. The oxidation step is decreased from ~2 h to less than 30 min in Ser65 → Thr mutants [11].

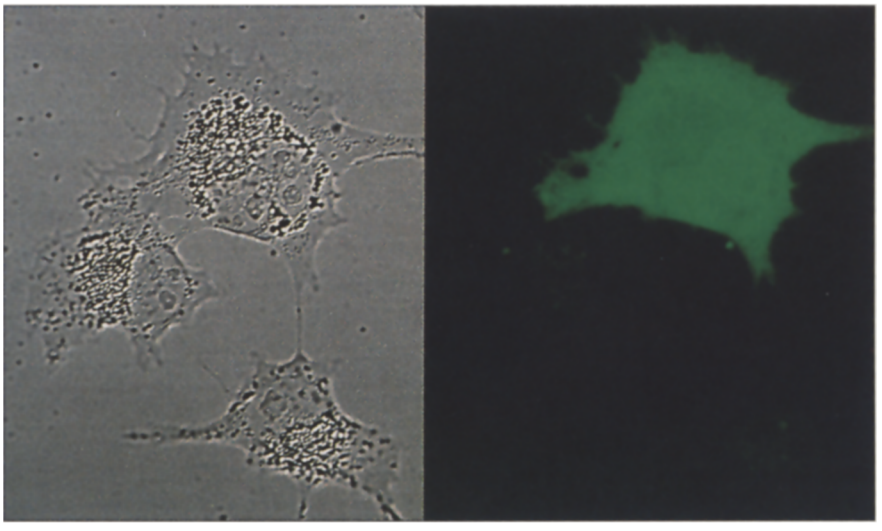

Fig. 2. Fluorescence of GFP in live mammalian cells. Laser-scanning confocal micrograph of live COS1 monkey cells transfected with a GFP expression construct. Left: phase contrast image. Right: fluorescein optics; image is a two-dimensional projection of a series of optical sections. The two non-fluorescent cells are not expressing GFP.

cDNA under control of the neuron-specific *mec7* promoter. We have shown that GFP is strongly fluorescent in live mammalian cells (Fig. 2). GFP appears to have no cytotoxic effect in the cell types in which it has been tested, and the protein partitions freely between the nucleus and cytoplasm (A.C. and T.H.B, unpublished data).

Many cellular processes can now, in principle, be studied in real time in living cells using GFP. A particularly exciting application of GFP would be to visualize protein trafficking. In the first experiment of this kind, GFP was expressed as a stable, fully-functional fusion protein in *Drosophila melanogaster*. The GFP cDNA was fused to the *exuperentia (exu)* gene, and the fusion protein was expressed in female *Drosophila* germ cells. The fusion protein fluoresced strongly during oogenesis, allowing the structures with which Exu protein associates to be identified. The fluorescence was found to be more intense than the immunofluorescence signal seen after staining with fluorescently-labeled anti-Exu antibodies [8]. Recently, a gene encoding a fusion protein of GFP linked to histone H2B was expressed in budding yeast; the fusion protein was found to localize to nuclei, as expected [9]. In neither study was there a detectable difference between the behavior of the native protein and that of the GFP fusion, indicating that GFP did not perturb the function of the fusion partner.

GFP is an almost ideal cell lineage tracer because of its stability, lack of cytotoxicity, and the fact that visualization is not subject to the artifacts associated with fixation, staining, or microinjection. These properties also make GFP an excellent reporter molecule in gene-trap experiments that are used to identify developmentally-regulated genes [10]. In these experiments, a promoterless reporter gene is allowed to integrate randomly into the genome of embryonic stem cells. The gene will only be expressed if it integrates into a transcription unit that is active in these cells or in their differentiated progeny. In the past, the *lacZ* gene, which encodes β-galactosidase, has been used for such experiments and expression of the gene product was detected by fixing and staining the cells. GFP will allow visualization in live specimens. Some of the obvious applications of GFP in biology are summarized in Table 1.

GFP mutants with new spectral properties

Although wild-type GFP has great advantages as a reporter molecule and a fluorescent protein tag, the protein does have limitations. Fluorophore maturation is

Table 1. Potential applications of GFP

Application	Advantages	Possible disadvantages
Reporter molecule in gene-expression studies	Real-time monitoring in live cells. Blue variant provides an internal control.	Slow oxidation step in fluorophore formation may limit time resolution. (Fast oxidizing mutant now available and even faster mutants may be obtainable.)
In vivo fluorescent tag	Real-time visualization of protein trafficking in live cells.	GFP may alter behavior of hybrid protein
Lineage marker, reporter in expression-trap studies	Allows direct visualization in intact cells and embryos.	No obvious disadvantages (provided GFP is fluorescent in all cell types).
Detection of protein–protein interactions by resonance energy transfer	Direct real-time measurement of protein interactions in live cells.	Sensitive to orientation of fluorophore dipoles and other constraints described by Forster equation.

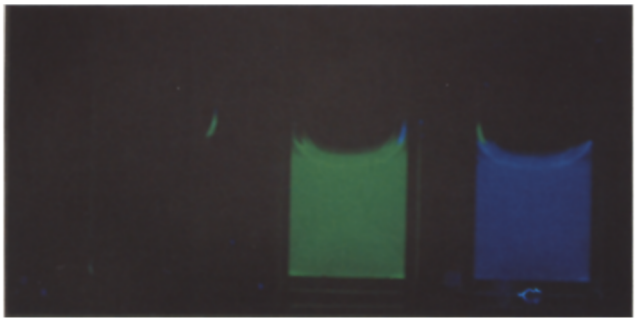

Fig. 3. A mutant GFP that fluoresces blue. *E. coli* transformed with left, vector alone; center, wild-type GFP; and right, mutant GFP (Tyr66 → His). Live bacteria were suspended in 1-cm cuvettes and illuminated at 365 nm [5]. Reprinted with permission from [5].

slow (~2 h) due to a lengthy oxidation step, and the more useful excitation maximum of 475 nm is of relatively low amplitude. Tsien and colleagues [11] have recently shown that a point mutation (Ser65 → Thr) in GFP can overcome both of these limitations. The oxidation step is much faster in the mutant protein than in the wild-type (less than 30 min compared to ~2 h), and when excited with 475-nm light, the light emitted by the mutant is approximately six-fold brighter than that emitted by the wild-type [11]. The same laboratory used a novel expression screen in *E. coli* to isolate additional mutants of GFP with altered spectral characteristics [5]. Mutant clones were identified as colonies that shone more brightly or in different hues upon illumination. A particularly useful mutant emits blue light (λ_{max} 448 nm) rather than green (λ_{max} 509 nm) when excited at 395 nm (Fig. 3). This mutant bears a single amino-acid substitution within the fluorophore (Tyr66 → His). Such variant forms of GFP will enable multiple gene-expression patterns to be monitored within the same cell simultaneously and, as suggested by Heim *et al.* [5], might allow the direct detection of protein–protein interactions via fluorescence resonance energy transfer.

Fluorescence resonance energy transfer

The transfer of excitation energy between two fluorophores may occur non-radiatively through dipole–dipole interactions. If the emission band of the donor fluorophore overlaps the excitation band of the acceptor fluorophore and other conditions of the Forster equation [6] are met, illumination in the donor excitation band will result in emission of light at the wavelength of the acceptor emission band. The efficiency of this resonance energy transfer decreases in proportion to the sixth power of the distance between the two fluorophores. Energy transfer is negligible when the distance is greater than 10 nm, and under physiological conditions will predominantly occur in cases of direct association between donor and acceptor. The GFP variants identified by Tsien and colleagues [5, 11] are suitable for resonance energy transfer experiments that would allow direct examination of the interaction of proteins fused to different fluorophores in live cells. This simple system may provide a new route to the identification of interacting proteins, and permit direct microscopic visualization of inducible protein–protein interactions, for example, those in signal-transduction cascades. Such an application is especially timely as it is now a simple matter to distribute time-lapse microscopy data over the internet in the form of QuickTime™ movies.

Future prospects

Although native GFP is already recognized as a power tool in cell biology, great improvements in the protein have already been made, and further modifications that will tailor GFP derivatives to specific applications are very likely to occur. Further mutations in the fluorophore may yield improvements, and the sensitivity of the characteristics of the emitted fluorescence to the local chemical environment in the folded protein may allow rational modification directed towards desired properties; a crystal structure would be very helpful for this purpose. Even in the unlikely event that no improvement is possible, the remarkable properties of the extant forms of GFP promises to revolutionize several areas of experimental biology.

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Angela Coxon, Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, 221 Longwood Avenue, Boston, MA 02115, USA and Timothy H Bestor, Department of Genetics and Development, College of Physicians and Surgeons of Columbia University, 701 West 168th Street, New York, NY 10032, USA.