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Invited Review

The molecular determinants of the efficiency of green fluorescent protein mutants

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Summary. The Green Fluorescent Protein (GFP) is a spontaneously fluorescent polypeptide of 27 kD from the jellyfish ***Aequorea victoria*** that absorbs UV-blue light and emits in the green region of the spectrum. GFP has been succesfully expressed both in bacteria and in eukaryotic cells and is widely used to monitor the localization of tagged proteins in living cells. Since wtGFP performs inefficiently in different cellular contexts, efforts have been devoted to the improvement of GFP expression levels and/or fluorescence. We will here review the basic characteristics of wt and mutated GFP, in particular their protein expression vs fluorescent properties. Emphasis will be given to unexpected consequences of mutations of the GFP gene, i.e. on transcription and translation rates and on protein folding in different cell types, and to how these critically reflect on the use of GFP in different cellular environments.

Key words: GFP, FRET, Fluorescence spectroscopy, Fluorescence microscopy

Introduction

The Green Fluorescent Protein (GFP) is a spontaneously fluorescent polypeptide of 27 kD (228 AA) from the jellyfish ***Aequorea victoria*** (Prasher et al., 1992; Chalfie et al., 1994; Inouye and Tsuji, 1994a; Cubitt et al., 1995; Prasher, 1995; Chiu et al., 1996; Heim and Tsien, 1996). In Aequorea, GFP converts the blue chemiluminescence of the photoprotein Aequorin into green fluorescence by energy transfer (Inouye and Tsuji, 1994a; Cubitt et al., 1995; Prasher, 1995; Kendall and Badminton, 1998). The GFP chromophore is generated by a cyclization of three adjacent aminoacids (S65, Y66, G67), followed by 1,2-dehydrogenation of the tyrosine (Heim et al., 1994; Inouye and Tsuji, 1994b; Cubitt et al., 1995; Ormo et al., 1996; Yang et al., 1996a; Youvan and Michel-Beyerle, 1996; Reid and Flynn, 1997). The chromophore alone is able to absorb light, but its fluorescent emission dependens on a protein 'sheath', an 11-stranded ß-barrel (Ormo et al., 1996; Yang et al., 1996a; Reid and Flynn, 1997). GFP has been succesfully expressed both in bacteria and in eukaryotic cells and has been produced by in vitro translation of the GFP mRNA (Chalfie et al., 1994; Inouye and Tsuji, 1994b; Prasher, 1995; Chiu et al., 1996; Heim and Tsien, 1996; Gubin et al., 1997; Kahn et al., 1997; Haseloff and Arnos, 1998; Pines, 1998). The latter indicates that the chromophore cyclization and folding (Makino et al., 1997) are, at least in a large part, autocatalytic and that GFP does not need specific cofactors to become fluorescent. GFP retains its fluorescence when fused to heterologous proteins (Cubitt et al., 1995; Laukkanen et al., 1996; Ludin and Matus, 1998; and submitted for publication). This property allows the use of GFP as a reporter for gene expression and to monitor the localization of GFP tagged proteins in living cells without the need for invasive labelling of the cells (Chalfie et al., 1994; Roessner and Scott, 1995; Anderson et al., 1996; Cheng et al., 1996; Chiu et al., 1996; Hampton et al., 1996; Andersen et al., 1998; Ludin and Matus, 1998).

In order to realize GFP-based multicolor analysis and to follow the interaction of GFP-tagged proteins by fluorescence resonance energy transfer (FRET), spectral variants of GFP have been produced that have different excitation and/or emission spectra (Heim et al., 1994, 1995; Cubitt et al., 1995; Delagrave et al., 1995; Ehrig et al., 1995; Anderson et al., 1996; Heim and Tsien, 1996; Ormo et al., 1996; Palm et al., 1997). This has also allowed a better match with available light sources, e.g. the blue line of argon-ion lasers (Heim et al., 1995; Cormack et al., 1996). Brighter mutants have also been seeked for to compensate for low expression levels in specific cellular environments (Anderson et al., 1996; Cormack et al., 1996; Crameri et al., 1996; Heim and Tsien, 1996; Siemering et al., 1996; Yang et al., 1996b, 1998; Zhang et al., 1996; Kimata et al., 1997). The large number of GFP variants has considerably complicated

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Table 1. Spectral properties and fluorescence values of major GFP variants.

GFP VARIANT	MUTATIONS	EXCITATION	EMISSION	ε	QY
wtGFP[1]	-	396(475)	508(503)	25000[a]/28000[b]	0.8
wtRenillaGFP[2]	-	498	509	135000	0.8
αGFP[3]	F99S-M153T-V163A	396	475	30000	0.8
S65T[4]	S65T	489	510	55000[a]/64000[b]	0.6[a]/0.66[b]
Bex1[5]	S65T-V163A	489	510	64000	0.6
Mut1[6]	S65T-F64L	489	510	55000	0.6
Vex-1[7]	S202F, T203Y, V163A	398	510	35100	0.8
YFP8	S65G-V68L-S72A-T203Y	513	527	36500	0.63
BFP(P4)[9]	Y66H	383	447	13500	0.21
BFP(P4-3)[9]	Y66H-Y145F	381	445	14000	0.38
E-BFP[10]	F64L-S65T-Y66H-Y145F	380	440	31500	≤ 0.20
CFP W7[9]	Y66W-N146I-M153T-V163A N212K	433	475	14000	0.63
ECFP[11]	Y66W, F64L, S65T, N146I, M153T, V163A	433(453)	475(501)	ND	ND

[1]: Wild type GFP (Chalfie et al., 1994; Siemering et al., 1996; Palm et al., 1997; Inouye and Tsuji, 1994a). Secondary excitation peaks are indicated in parenthesis. Values are reported as in Patterson et al. (1997)[a] or in Ciccocioppo et al. (submitted)[b]. [2]: Values as reported in (Cubitt et al., 1995). [3]: αGFP is the Cycle 3 mutant reported by Crameri et al. (1996). The commercially available GFPuv is a Cycle 3 mutant codon-optimized for expression in mammalian cells. Values as reported in Patterson et al. (1997). [4]: Values as reported in Patterson et al. (1997) ([a]) and in Ciccocioppo et al. (submitted) ([b]). [5]: Values as reported in Ciccocioppo et al. (submitted). [6]: Values reported by Patterson et al. (1997) and Yang et al. (1998). EGFP is codon-optimized for mammaliam cells (Yang et al., 1996b). [7]: Values as reported in Ciccocioppo et al. (submitted). [8]: Values reported in Ormo et al. (1996) for the mutant 10C. [9]: Values as reported in Heim and Tsien (1996). [10]: Values as reported in Patterson et al. (1997) and Yang et al. (1998). [11]: Values as reported by Clontech.

their analysis and it is often unclear to what extent their properties have been verified. In particular, comparison with inefficiently expressed wtGFP and insufficient discrimination between efficiency of expression vs fluorescence of mutGFP have often overestimated the real improvements of mutGFP over wtGFP (submitted for publication). Recently, we have demonstrated that specific prokaryotic-selected mutants can perform quite poorly in mammalian cells (submitted for publication). Thus, accurate comparison in one environment may not hold true in another one.

In this review, we will focus on the basic characteristics of wt and mutated GFP, and how these may critically reflect on the use of GFP in different cellular environments.

GFP fluorescence and spectral variants

The Aequorea GFP possesses a major excitation peak at 396 nm and a minor one at 475 nm. The emission peaks at 507 nm, if excited at 396 nm, or at 503 nm, if excited at 475 nm (Table 1, Fig. 1) (Ward et al., 1982; Cubitt et al., 1995; Heim and Tsien, 1996; Brejc et al., 1997; Patterson et al., 1997 and unpublished observations). The two different excitations correspond to protonated (396 nm) and deprotonated (475 nm) tyrosine 66, that can transfer its hydrogen to Glu-222 (Chattoraj et al., 1996; Brejc et al., 1997). The two different chromophore forms are in a ratio of about 6:1 (Brejc et al., 1997).

Remarkably, the fluorescence of wt and blue-excited GFP can be excited with some efficiency at 280 nm (Fig. 1). The corresponding emission peaks at 385 nm and at the canonical wavelength of 507 nm. This suggests light absorption by Y66 and either radiative FRET or non-radiative transfer to the tricyclic GFP chromophore. We favor the latter, since wt and Bex1 demonstrate a roughly equal efficiency, but Bex1 does not possess a UV-absorbing chromophore, i.e. a suitable acceptor for FRET. Mutant GFP with different emission colors have been obtained, e.g. blue (BFP) (Heim et al., 1994; Heim and Tsien, 1996; Wachter et al., 1997; Yang et al., 1998), cyan (CFP) (Heim et al., 1994; Heim and Tsien, 1996), and yellow (YFP) (Ormo et al., 1996; Wachter et al., 1998). Mutant GFP with different excitation spectra have also been obtained, e.g. blue-excitation, for mutants in S65 (Anderson et al., 1996; Cormack et al., 1996), or violet excitation for T203I mutants (Heim et al., 1994; Ehrig et al., 1995; Anderson et al., 1996).

Blue emission has been obtained by mutating the Y66 in the chromophore to H (Heim et al., 1994; Heim and Tsien, 1996; Wachter et al., 1997). These mutants, are excited at about 380 nm and emit around 450 nm (Table 1). CFPs result from a Y66W mutation and possess an excitation maximum at 440 and emission at 480 nm (Table 1) (Heim et al., 1994; Heim and Tsien, 1996). YFP have been generated by further mutating T203 to H or Y in different S65 mutants. In some of them, additional aminoacidic changes are reported to be important in improving protein folding and consequently

GFP fluorescence (Heim and Tsien, 1996). The most red shifted YFP is the 10C mutant (Ormo et al., 1996), that absorbs at 513 nm and emits green-yellow light, with a maximum at 527 nm (Table 1) (Ormo et al., 1996). Other YFP spectra are very close to those of 10C GFP (Ormo et al., 1996).

Blue-excited green-emitting mutants have been generated by substituting S65 with different aminoacids (Cubitt et al., 1995; Delagrave et al., 1995; Heim et al., 1995; Cormack et al., 1996). The blue excitation is a consequence of a permanently deprotonated chromophore (Brejc et al., 1997). The best characterized and most widely used are the S65T mutants (S65T, mut1/EGFP, Bex1) (Table 1) (Anderson et al., 1996; Patterson et al., 1997; Yang et al., 1998).

The T203I aminoacidic substitutions produce a flattening of the secondary excitation peak (Heim et al., 1994; Ehrig et al., 1995), that is consequent to the prevalence of the neutral chromophore form. Vex1 (S202F, T203I, V163A) is excited at 398 nm, but almost totally lacks the secondary excitation peak of wtGFP (Fig. 1) (Heim et al., 1994; Anderson et al., 1996; and submitted).

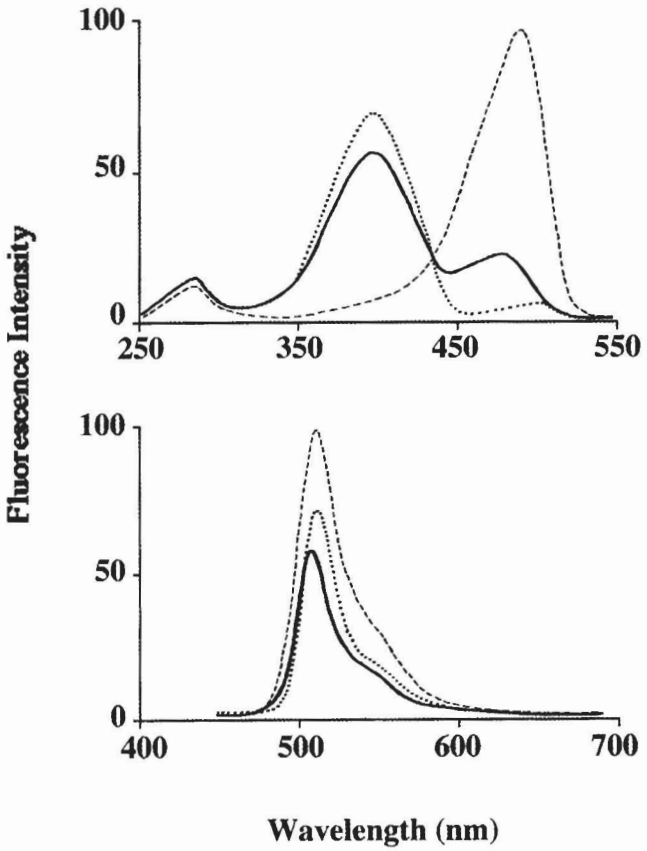

Fig. 1. Fluorescence spectra of major GFP variants. **Top.** Excitation spectra. **Bottom.** Emission spectra. Solid line: wt; dotted line: Vex1; dashed line: Bex1. The height of the peaks is drawn in proportion to the respective optical efficiency, i. e. QY times e, of each GFP variant.

Green, yellow, cyan and blue GFP variants are a useful tool for multi-color analysis (Heim and Tsien, 1996; Ormo et al., 1996; Rizzuto et al., 1996; Kendall and Badminton, 1998; Yang et al., 1998). The number of different events that can be followed simultaneously can be increased by using different excitation wavelengths, e.g. violet and blue light nm for Vex1 and S65T mutants (Anderson et al., 1996). Blue/cyan emitting mutants and the blue excited S65 mutants or green excited YFPs can be used to follow the physical interaction of GFP-tagged proteins by energy transfer (Kendall and Badminton, 1998; Mahajan et al., 1998; Xu et al., 1998). Vex1 can be used in combination with S65T mutants in dual excitation analysis (Anderson et al., 1996).

A naturally existing variant of GFP has been obtained from the sea pansy *Renilla reniformis* (RGFP) (Cubitt et al., 1995; Prasher et al., 1992). RGFP possesses a single excitation peak at 498 nm (Table 1). Its strong tendency to aggregate, however, has not encouraged its use. A variety of different fluorescent proteins of potential interest are present in other Cnidaria, but require further characterization (Ward et al., 1982; Prasher et al., 1992; Prasher, 1995).

Fluorophore efficiency

The efficiency of use of a chromophore critically depends on how much light it absorbs (extinction coefficient (ε) and what fraction of the absorbed energy is reemitted as light (quantum yield, QY).

Extinction coefficient

The GFP possess an ε_{396} of 25000-28000 $mol^{-1}cm^{-1}$ and an ε_{475} of 9500-11000 $mol^{-1}cm^{-1}$ (Patterson et al., 1997; and submitted for publication). The two different excitations correspond to the protonated and deprotonated chromophores. Thus, the higher ε_{396} results from a higher fraction of protonated chromophore, the effective ε_{475} being about twofold higher than the ε_{396} (Brejc et al., 1997).

Only slightly better than the wild type molecule is αGFP, a mutant with the same spectra as wtGFP (Crameri et al., 1996), and Vex1 (Anderson et al., 1996), with ε of 30000 and 35000 $mol^{-1}cm^{-1}$, respectively (Table 1) (Patterson et al., 1997; and submitted). Significant improvement has been obtained with the S65T mutants, that demonstrate an ε_{489} of 55000-64000 (Table 1) (Delagrave et al., 1995; Brejc et al., 1997; Patterson et al., 1997; and submitted). The YFP mutant 10C has an ε_{513} of 36500 (Ormo et al., 1996). Other yellow mutants are worse, probably because a significant fraction of YFP is non fluorescent (see below). The Y66H mutation produces a BFP chromophore with an ε lower than wtGFP (Table 1) (Heim and Tsien, 1996). More efficient are the Y66W CFPs (Heim and Tsien, 1996). Quite a high ε (about 135.000 $cm^{-1}mol^{-1}$ for the monomer), has been reported for RGFP. It is interesting to note that the chromophore of RGFP is the same cyclic

tripeptide of Aequorea GFP, but a different aminoacidic environment allows an apparently much higher absorption efficiency. No GFP variants with comparable ε are available yet. Some reported high ε have not been confirmed by subsequent measurements and probably resulted from comparison with inadequate wtGFP standards (Yang et al., 1996b).

Quantum yield

wtGFP and RGFP have a QY of about 0.8, i. e. they are very efficient in reemitting light (Cubitt et al., 1995; Heim and Tsien, 1996; Patterson et al., 1997). GFP spectral mutants possess a lower QY (Patterson et al., 1997), with the exception of Vex1 GFP, that possesses a QY of 0.8 (Anderson et al., 1996) (Table 1).

Fluorescence efficiency

The product between ε and QY quantifies the efficiency of a given fluorophore (Yang et al., 1998). The S65 mutants are overall more efficient than the wt molecule. The improvement in fluorescence emission of the S65T mutant over the wtGFP, is of 1.7-1.9 fold, if both GFP are excited at their optimal wavelength, but it goes up 6-8 fold if both are excited at 488 nm (Table 1) (Cubitt et al., 1995; Patterson et al., 1997; and submitted). Vex1 is 1.3 fold more efficient than wtGFP (submitted). BFPs and CFPs are less efficient than the wt (Table 1), and this has limited their use.

Problems in determining ε and QY

Since only the correctly folded protein produces a functional chromophore, the accuracy of ε determination using Beer's law is negatively affected by the coexistence of non absorbing/non fluorescent GFP. This is particularly relevant at 37 °C (Cubitt et al., 1995; Siemering et al., 1996; Patterson et al., 1997; and submitted), since the physiological environment of *Aequorea victoria* is at fairly low temperatures (Prasher et al., 1992), and wtGFP has a suboptimal folding at higher temperatures (Cubitt et al., 1995; Ogawa et al., 1995; Siemering et al., 1996; Patterson et al., 1997; and submitted). Accurate measurments of ε can be obtained by comparison with the absorption characteristics of the sample after a complete denaturation of GFP, e. g. by alkali (Fig. 2) (Cody et al., 1993; Patterson et al., 1997). Totally denatured GFP still absorbs light through its chromophore (a covalently closed structure), but is unable to reemit it by fluorescence. The absorption maximum of the "naked chromophore" is at 448 nm (Fig. 2), with an ε of 44100 $mol^{-1}cm^{-1}$, and this can be used as an internal standard of molar absorption. Y66H and Y66W mutants do not show a 448 nm absorption peak upon alkaline denaturation. The determination of appropriate reference values might prove useful, and allow a reevaluation of their published ε. In general, estimated values of ε that are not based on the 448 nm reference are probably underestimated.

Trivial factors can also affect the accurate determination of ε. Extinction coefficients are molar, i.e. they require a correct measurement of GFP concentration. In our hands, Biorad assays in solutions proved unreliable, whereas good results have been obtained with BCA assays. Blue Coomassie staining in gels also proved adequate for semi-quantitative evaluation. A theoretical ε_{280} of GFP of 19890 mol^{-1} cm^{-1} can be calculated from sequence data (Gill and von Hippel, 1989), and fits well experimental BCA assay estimates. It should be noted that sequence tags added to GFP can markedly affect the absorption at 280 nm, and should be taken into account. Estimates of GFP concentration of homogenously purified samples by absorption at 280 nm can also suffer from spectral interference by the imidazol buffer commonly used for the elution of GFP from Ni-chelate columns. We have also observed that freezing-thawing of blue-excited GFP artefactually reduces their ε (see, for example, Table 1, notes 1a vs 1b). Thus, care should be taken in measuring the actual ε in freshly prepared samples.

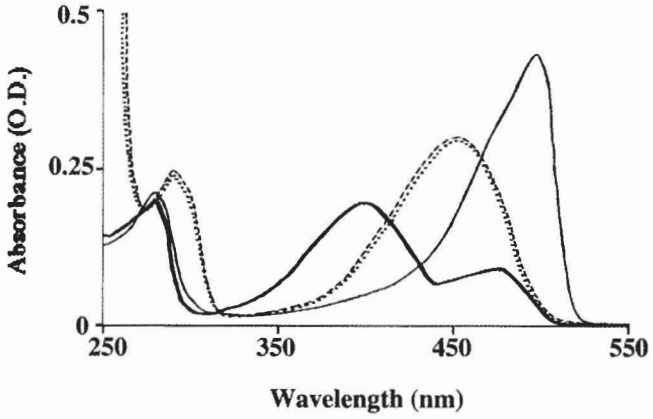

Fig. 2. Absorbance spectra of wt and Bex1 GFP. Thick solid line: wt; thin solid line: Bex1; dotted line: alkaline denatured wt; dashed line: alkaline denatured Bex1.

Spectral perturbations

High levels of wtGFP within cells alter its excitation spectrum. The favored formation of GFP dimers causes a flattening of the 475 nm excitation peak (Ward et al., 1982; Cubitt et al., 1995; and unpublished observations). As a consequence, a 396/475 excitation ratio of about 2.5-3 is observed in cells with a low GFP concentration, whereas a ratio of 6 is observed in the presence of GFP dimers (Ward et al., 1982; Cubitt et al., 1995; and unpublished observation). This may render non linear the quantification of GFP fluorescence among intact cells if 488 nm light is used for excitation.

Chromophore photobleaching

wtGFP is characterized by a slow photobleaching if excited at 475 nm (Cubitt et al., 1995; Patterson et al.,

1997). UV/violet stimulation, on the other hand, produces a very fast photoconversion that overlaps with a much slower photobleaching (Cubitt et al., 1995; Chattoraj et al., 1996; Youvan and Michel-Beyerle, 1996; Brejc et al., 1997; Patterson et al., 1997). This ends up in a reduced absorption at 396 nm, and an increased one at 475 nm. The latter is due to the production of a deprotonated chromophore (Forster cycle) (Chattoraj et al., 1996; Youvan and Michel-Beyerle, 1996; Brejc et al., 1997), and is reversible after recovery in the dark (Brejc et al., 1997; Patterson et al., 1997). Photoconversion represents a problem in the use of wtGFP in optical microscopy if UV/violet stimulation is used, but is essentially irrelevant in flow cytometry, because of the much shorter illumination time. The 475 nm excitation peak is highly stable, and only shows a slow photobleaching (Patterson et al., 1997). The S65 mutants undergo only slow photobleaching upon blue light illumination (Cubitt et al., 1995; Patterson et al., 1997). BFP, on the other hand, does not undergo photoconversion, but is rapidly photobleached (Patterson et al., 1997; Wachter et al., 1997).

Resistance to pH

The wtGFP fluorescence is stable in the range of pH 6-10, and is even increased at pH 10-12. On the other hand, GFP rapidly loses its fluorescence at pH below 6 (Ward et al., 1982; Patterson et al., 1997). A similar behaviour is showed by αGFP (Patterson et al., 1997) and Vex1 (unpublished observation). The S65T and BFP mutants are less resistant at acidic pH than wtGFP (Patterson et al., 1997). These problems may limit the use of wtGFP, and even more that of specific GFP mutants, in acidic subcellular compartments. However, this can also be usefully exploited to follow pH decrease in living cells during the transit of GFP in different cellular compartments (Llopis et al., 1998; Miesenbock et al., 1998; Robey et al., 1998; Wachter et al., 1998). New GFP mutants have been produced to obtain a better resolution of pH changes (Miesenbock et al., 1998). Interestingly, a mutant called 'ratiometric pHluorin' shows a different ratio of 396/475 excitation when the pH goes below 7 (Miesenbock et al., 1998). This is a distinct improvement over GFP forms that only undergo loss of fluorescence at acidic pH.

Efficiency of protein folding

Since the formation of a GFP chromophore is an autocatalytic process, it probably both dictates and requires a correct folding of the protein (Cubitt et al., 1995; Patterson et al., 1997). The wtGFP is largely incorrectly folded if produced at 37 °C in bacteria, with a 60-90% of protein that is accumulated in the insoluble fraction, and 70-80% of soluble protein that is inactive (Ogawa et al., 1995; Siemering et al., 1996; Patterson et al., 1997 and submitted). The fluorescent protein, on the other hand, is not temperature sensitive, since GFP produced at RT remains fluorescent for several hours after switching to 37 °C (Siemering et al., 1996).

GFP mutants that are brighter as a consequence of a better folding at high temperatures have been produced. Useful to this purpose are the V163A and the F64L aminoacidic substitution. Indeed, mut1GFP (Cormack et al., 1996; Patterson et al., 1997) and Bex1 (Anderson et al., 1996 and submitted) perform considerably better than S65T-GFP both in bacteria and in eukaryotic cells (submitted). Enhanced mutants of CFP and BFP, named ECFP and EBFP, also result from the F64L substitution (Table 1). CFP also contains the V163A mutation. Similarly, the introduction of V163A in Vex1-GFP has improved its previously dim fluorescence (Heim et al., 1994; Anderson et al., 1996; and submitted). GFP tags can also affect protein folding. Thus, care should be taken in quantitating tagged GFP vs native GFP.

Protein expression in eukaryotic cells

The final levels of GFP that can be obtained clearly are critical to its use. The steady state levels of GFP, like for any other protein, are determined by the transcription and degradation rates of the GFP mRNA and by the translation vs degradation rates of the protein (submitted).

Since the first attempts of GFP subcloning in mammalian expression vectors, strong promoters proved useful for efficient GFP expression (Cubitt et al., 1995). Interestingly, some of the vectors used, for example the HTLV-1-driven pBJI-neo, albeit quite efficient in several other contexts proved quite poor in GFP expresssion (submitted).

Kozak- and codon-optimized mutants (Yang et al., 1996b, 1998; Haseloff et al., 1997) have been produced by silent mutations to improve GFP expression levels. It is unclear however to what extent these mutations have really improved GFP expression, since a separate assessment of fluorescence vs. protein levels is often missing. Interestingly, an up to 3-fold difference in protein steady state levels is caused by GFP mutations. For example, Vex1 is accumulated at one third of the levels of wtGFP-C (Ciccocioppo et al., submitted). The addition of aminoacidic tags can cause similar effects.

Notably, the removal of 5' untranslated (UT) region enormously improves the translation efficiency of GFP mRNA, albeit causing a reduction of its transcription rate (submitted). A similar effect is obtained by introducing GFP sequence tags (submitted). Kozak sequences do not prove to be a critical variable since the highest protein translation rate is shown by a wtGFP devoid of the 5' UT and that possesses the native sub-optimal GFP Kozak sequence (submitted). Taken together, our results indicate that alterations of the GFP open reading frame and of the surrounding sequences deeply affect the transcription and translation ability of GFP and are crucial to the overall efficiency of GFP mutants.

Conclusions

GFP is widely used as a reporter for gene expression and to monitor the localization of GFP-tagged proteins in living cells. Several mutant GFPs are now available and their use is becoming more and more sofisticated and specialized. Care should be taken, though, in the evaluation of the basic properties of the different mutants, particularly when quantitative measurements are sought for or high levels of correctly folded protein are required.

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