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Plasmids for C-terminal tagging in *Saccharomyces cerevisiae* that contain improved GFP proteins, Envy and Ivy

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Abstract

Green Fluorescent Protein (GFP) has become an invaluable tool in biological research. Many GFP variants have been created that differ in brightness, photostability, and folding robustness. We have created two hybrid GFP variants, Envy and Ivy, which we placed in a vector for the C-terminal tagging of yeast proteins by PCR-mediated recombination. The Envy GFP variant combines mutations found in the robustly folding SuperfolderGFP and GFP γ , while the Ivy GFP variant is a hybrid of GFP γ and the yellow-green GFP variant, Clover. We compared Envy and Ivy to EGFP, SuperfolderGFP, and GFP γ , and found that Envy is brighter than the other GFP variants at both 30°C and 37°C, while Ivy is the most photostable. Envy and Ivy are recognized by a commonly used anti-GFP antibody, and both variants can be immunoprecipitated using the GFP TRAP *Camelidae* antibody nanotrap technology. Because Envy is brighter than the other GFP variants and is as photostable as GFP γ , we suggest that Envy should be the preferred GFP variant, while Ivy may be used in cases where photostability is of utmost importance. The GenBank accession number for Envy is KM891731, Ivy is KM891732, and the yeast optimized GFP γ described in this paper is KM891733.

Keywords

green fluorescent protein; bright; photostable; folding optimized; fast-maturing; yeast vector

INTRODUCTION

Since the discovery of Green Fluorescent Protein (GFP) in the jellyfish *Aequorea victoria* [Shimomura *et al.*, 1962], followed by the subsequent cloning of the gene [Prasher *et al.*, 1992] and the demonstration of its heterologous expression in model organisms [Chalfie *et al.*, 1994; Inouye and Tsuji 1994a], GFP has become a versatile and ubiquitous tool in the biological sciences. As GFP requires no co-factors other than molecular oxygen for its activation [Inouye and Tsuji 1994b; Heim *et al.*, 1994], it is an ideal tool for the study of gene expression and protein localization in live cells [Shaner *et al.*, 2005]. The ease of creating fusion proteins in yeast via one-step PCR-mediated recombination [Longtine *et al.*, 1998; Lee *et al.*, 2013] has greatly facilitated protein localization studies [Huh *et al.*, 2003].

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Protein technologies for high affinity isolation of GFP fusion proteins [Rothbauer *et al.*, 2008] have expanded the versatility of GFP as a tool for biochemical studies.

wtGFP from *Aequorea victoria* has been modified for qualities desirable in biological research. The S65T mutation was the first change, which significantly increased brightness [Heim *et al.*, 1995]. EGFP (enhanced green fluorescent protein), another GFP variant with improved performance at 37°C, followed soon afterward [Cormack *et al.*, 1996]. As yeast are mainly grown at 30°C, the original S65T variant has been used extensively in yeast research [Longtine *et al.*, 1998].

Other desirable characteristics have also been engineered into GFP. Fast and robustly folding GFP variants have been created, such as SuperfolderGFP [Pédalacq *et al.*, 2006]. SuperfolderGFP was optimized for rapid folding, creating a GFP variant with a quickly maturing chromophore [Pédalacq *et al.*, 2006]. The yellow-green GFP variant, Clover, was engineered for quantum yield, photostability, and enhanced performance in FRET experiments. These alterations resulted in a shift of its excitation and emission peaks relative to EGFP [Lam *et al.*, 2012]. GFP γ was originally constructed for use in *C. albicans* and displayed exceptional brightness and photostability [Zhang and Konopka, 2010]. GFP proteins have also been codon-optimized for use in different model organisms to enhance their translation [Lee *et al.*, 2013].

In this study, we describe two new GFP variants, Envy and Ivy. Envy is a hybrid of the mutations in SuperfolderGFP [Pédalacq *et al.*, 2006] and the photostable yeast codon-optimized GFPgamma [Zhang and Konopka, 2010; Lee *et al.*, 2013], while Ivy is a hybrid of GFP γ [Zhang and Konopka, 2010] and the yeast codon-optimized version of Clover [Lam *et al.*, 2012; Lee *et al.*, 2013]. Envy and Ivy combine the brightness and photostability described in GFP γ with the desirable properties seen in SuperfolderGFP and Clover, respectively.

MATERIALS AND METHODS

Plasmids

Plasmids used in this study are listed Table 1. Primers are listed in Table 3. Construction details are described below. All PCR reactions were performed using Phusion High-Fidelity DNA Polymerase (New England Biolabs). The sequences of Envy and Ivy were verified by sequencing. The yeast-optimized GFP variants yoEGFP, yoClover, yoSuperfolderGFP, and yoGFPgamma [Lee *et al.*, 2013] were obtained from Addgene and used to construct plasmids: pFA6a-link-yoGFP γ -SpHis5 (Addgene plasmid 60781), pFA6a-link-Envy-SpHis5 (Addgene plasmid 60782) and pFA6a-link-Ivy-SpHis5 (Addgene plasmid 60783). These plasmids can be obtained from Addgene.

When we obtained yoGFPgamma from Addgene, we sequenced the GFP portion of the plasmid and found that it had a phenylalanine at residue 64. This is unlike the original version of GFP γ [Zhang and Konopka, 2010] or EGFP [Cormack *et al.*, 1996], both which have a leucine at residue 64. We corrected residue 64 to replace it with the leucine present in the original GFP γ (and EGFP) via site-directed mutagenesis on yoGFPgamma using

OLH1501 and OLH1502, and sequenced the resulting DNA. We refer to the corrected version as yoGFP γ , as it is the version of yeast optimized GFP γ with the same amino acids as the original GFP γ [Zhang and Konopka, 2010].

pFA6a-link-Ivy-SpHis5 and pFA6a-link-Envy-SpHis5 were each created in two steps. For pFA6a-link-Ivy-SpHis5, pFA6a-link-yoClover-SpHis5 [Lee *et al.*, 2013] was mutagenized using primers OCS7 and OCS8 to introduce the following mutations: F64L, G65C and S72A; this resulted in the intermediate pFA6a-link-yoClover(III)-SpHis5. pFA6a-link-yoClover(III)-SpHis5 was then mutagenized with primers OCS9 and OCS10 to add the I167T mutation, which produced pFA6a-link-Ivy-SpHis5. To create pFA6a-link-Envy-SpHis5, pFA6a-link-yoSuperfolderGFP-SpHis5 [Lee *et al.*, 2013] was mutagenized using primers OCS3 and OCS4, to add the mutations T65C and S72A, creating the intermediate, pFA6a-link-yoSuperfolderGFP(II)-SpHis5. pFA6a-link-yoSuperfolderGFP(II)-SpHis5 was then mutagenized with primers OCS5 and OCS6, to add the mutation I167T, to create pFA6a-link-Envy-SpHis5.

Yeast strains used in this study

All yeast strains used in this study were derived from the SK1 background [Huang *et al.*, 2005] and listed in Table 2. C-terminal tagging was accomplished by PCR mediated recombination [Longtine *et al.*, 1998; Puig *et al.*, 1998]. The expression of fusion proteins was confirmed by fluorescent microscopy and Western blot analysis. All strains in Table 2 were constructed using the primer pair OCS1 and OCS2 and the appropriate GFP variant plasmid.

Yeast Growth Conditions

Yeast cells used for brightness and photostability measurements were grown overnight in YPD (2% Peptone, 1% Yeast Extract, 2% Dextrose) at either 30°C or 37°C, as indicated, until an OD₆₀₀ between 3.0 and 4.0. Cells grown for Western analysis to test the JL-8 antibody and for immunoprecipitation of Bmh2 were grown overnight in YPD, diluted back to an OD₆₀₀ of 0.1, and then collected at an OD₆₀₀ between 0.6 and 0.8.

Fluorescence microscopy

Microscopy was performed using a 100x (NA 1.45) objective on a Zeiss Axioskop Mot2. Images were taken using an Orca-ER cooled CCD camera (Hamamatsu) using Openlab 4.04 (Perkin Elmer) for image acquisition. All imaging was done using live cells. All strains were observed using Zeiss filter 38, which has an excitation center wavelength of 470 nm and bandwidth 40 nm.

Calculating brightness levels

Image analysis was performed using ImageJ [Schneider *et al.*, 2012]. Single cells were selected, fluorescence intensity was measured, and three cell-free selections were made around each cell to subtract out average background fluorescence, as previously described [Burgess *et al.*, 2010]. Fluorescence intensity measurements were collected over multiple days. For a single experiment, all GFP variants (EGFP, SuperfolderGFP, GFP γ , Envy, and Ivy) were all measured on the same day. Brightness levels were calculated by normalizing

the fluorescence intensity of the GFP variant divided by the level of fusion protein (as assessed by Western analysis), and normalized to the fluorescence intensity of EGFP (measured on the same day as the GFP variant) divided by the level of EGFP protein, to correct for differences in lamp strength and intensity over time. Samples for Western analysis were carried out on cells from the same culture that fluorescence intensity measurements were obtained.

Measurements of photostability

Photostability measurements were calculated by taking pictures every 10 seconds over the time span of the experiment while cells were under continuous fluorescent illumination. Initial cell brightness was determined using the method described above. Each subsequent measurement was normalized to the initial brightness for each cell. Photostability data for each strain were taken on at least three different days to control for day-to-day fluctuation in cell fluorescence and lamp brightness. Normalization of brightness to the initial image also compensates for the differences in GFP variant fusion protein levels that might occur in each cell.

Protein immunoblotting

Protein lysates for immunoblotting were prepared using trichloroacetic (TCA) precipitation, as described [Philips and Herskowitz, 1998]. After TCA precipitation proteins were re-suspended in SDS-PAGE sample buffer [Huang *et al.*, 2005] boiled for 5 minutes and separated by SDS-PAGE. Proteins were transferred onto Polyvinylidene fluoride (GE Healthcare) and probed with the following antibodies: mouse monoclonal JL-8 (Takara Clontech) at 1:1000 to detect GFP, mouse monoclonal 22C5D8 (abcam) at 1:1000 to detect Pgl1, and rabbit polyclonal anti-Bmh [Gelperin *et al.*, 1995] at 1:10000 to detect Bmh1 and Bmh2 fusion proteins.

Blots were visualized on the Odyssey CLx Infrared Imaging System (LI-COR Biosciences), blocked with Odyssey blocking buffer (LI-COR), and incubated with either Donkey anti-Mouse IRDye800CW (LI-COR) or Donkey anti-Rabbit IRDye800CW (LI-COR) secondary antibodies, at 1:10000 as appropriate. Protein bands were quantified using Image Studio (LI-COR).

Immunoprecipitation

Lysates for immunoprecipitation were prepared from 20 OD₆₀₀ of cells. Cell pellets were lysed in a MiniBeadBeater8 (Biospec) at 4°C with glass beads in IP buffer (300mMNaCl, 5 mM EGTA (pH 8.0), 50 mMTris (pH 7.4), and 0.5% Nonidet P-40) with added protease and phosphatase inhibitors as previously described [Huang *et al.*, 2005].

Lysate was clarified with three spins using a tabletop microcentrifuge (Eppendorf). Clarified lysate was then added to 40µl of blocked agarose beads (Bulldog Bio.) incubated on a nutator at 4°C for 30 minutes. Blocked agarose beads were removed from the lysates by gentle centrifugation. GFP-Trap A beads (Bulldog Bio) were then added to the lysate, which was incubated on a nutator at 4°C for 2 hours with 20µl of GFP-Trap A beads (Bulldog Bio).

GFP-Trap complexes were washed 4 times in IP buffer and re-suspended in 2X SDS-PAGE sample buffer, boiled for 5 minutes and then separated by SDS-PAGE.

RESULTS

Creation of the GFP Hybrids, Envy and Ivy

Recent studies report that EGFP is the best overall green fluorescent protein in terms of brightness and photostability when imaging in yeast [Lee *et al.*, 2013]. As the brightness and maturation rate of a fluorescent protein affects its ability to be visualized, especially when fused to proteins that are short-lived or expressed at low levels, we wanted to create GFP variants that would maximize these parameters. To achieve this goal, we chose to incorporate the GFP γ mutations into the SuperfolderGFP and Clover backgrounds (Figure 1 and Table 1).

Due to overlap in mutations between SuperfolderGFP and GFP γ , only three mutations were required to change SuperfolderGFP into GFP γ Envy (Envy): T65C, S72A and I167T. To place the relevant GFP γ mutations into the Clover background required mutation of four residues: F64L, G65C, S72A and I167T. We called this hybrid GFP γ Ivy (Ivy). Clover has a valine after the initial methionine so that the translational start sequence is optimized [Shaner, Steinbach, and Tsien 2005]. This valine is not shown in Figure 1, nor do we include it when numbering residues in this paper, so that the numbering of Clover and Ivy residues will be aligned with other GFP variants (Figure 1).

To create Envy and Ivy, we started with the yeast optimized SuperfolderGFP and the yeast optimized Clover, respectively. These yeast-optimized GFP variants were in the pFA6a backbone, designed to facilitate C-terminal protein tagging [Sheff and Thorn 2004; Lee *et al.*, 2013]. Because we engineered Envy and Ivy within this vector backbone, Envy and Ivy can be used to C-terminally tag proteins in yeast (Figure 2A). Using PCR, a piece of DNA that includes the GFP variant, the terminator from the *ADHI* locus, and the *S. pombe His5* selectable marker (which can complement the *S. cerevisiae his3* mutations), can be amplified and used for tagging by PCR-mediated recombination (Figure 2B).

Envy is the Brightest Fluorescent Protein Tested

To compare the brightness of our hybrid GFPs with the parent GFP variants, we created Bmh2 GFP fusion proteins and examined fluorescence intensity to calculate brightness, as described in Materials and Methods. Bmh2 is a ubiquitously expressed protein with both nuclear and cytoplasmic localization [Tkach *et al.*, 2012; Slubowski *et al.*, 2014]. We examined Bmh2-EGFP, Bmh2-SuperfolderGFP, Bmh2-GFP γ , Bmh2-Ivy, and Bmh2-Envy. We did not include the GFP variant Clover in our analysis, as we saw weak signal from Bmh2-Clover under our assay conditions. The excitation peak of Clover (505nm [Lam *et al.*, 2012]) is incompatible with the filter used, which is optimized for GFP detection (excitation center wavelength of 470nm and bandwidth 40nm).

Our comparisons of brightness show that at 30°C, Envy was more than twice as bright as EGFP, and significantly brighter than GFP γ (p -value 2.59×10^{-15} ; Students t test)(Figure 3 and Table 4). Ivy was found to be comparable to EGFP under the conditions tested.

We also examined the brightness of the fusion proteins at 37°C. We reasoned that brightness might be enhanced at an increased temperature due to an increase in folding efficiency. An increase in folding efficiency of GFP could greatly impact the ability to visualize proteins with short half-lives or that fold poorly. We were also interested in determining how Envy and Ivy would behave at a temperature relevant to other experimental systems, such as mammals and bacteria. At 37°C, Envy was nearly 4-fold brighter than EGFP and 2-fold brighter than GFP γ (Figure 3 and Table 4). Ivy was slightly brighter than EGFP at 37°C, although to a lesser degree than the other GFP variants tested (Figure 3 and Table 4).

Ivy is the Most Photostable Fluorescent Protein Tested

As Envy successfully incorporated the superior brightness of GFP γ into the SuperfolderGFP background, we tested whether our hybrids also displayed increased photostability. When fused to Bmh2, Ivy yielded the most photostable product (Figure 4 and Table 5). After 60 seconds of continuous illumination by the fluorescent lamp, SuperfolderGFP and EGFP derivatives retained <15% of their initial brightness. In contrast, Ivy retained ~74% of its initial brightness after 60 seconds and ~50% after 120 seconds. Envy was also significantly more photostable than EGFP and Superfolder, retaining ~60% of its initial brightness after 60 seconds and ~40% after 120 seconds (Figure 4 and Table 5). Ivy's photostability also exceeded that of GFP γ , which was similar to that of Envy (Figure 4 and Table 5).

Envy and Ivy Are Compatible with Protein Analysis Tools

In addition to being a useful tool for cell imaging studies, GFP is an important tool for biochemical analysis. GFP antibodies allow for the detection of GFP fusion proteins on immunoblots, and the GFP-trap technology facilitates affinity-purification of GFP fusion proteins [Rothbauer *et al.*, 2008].

To determine whether Envy and Ivy were compatible with these technologies, we performed Western blot analysis of the strains used in this study (Figure 5). We probed our membrane with the JL-8 monoclonal antibody and found no difference in the ability of JL-8 to recognize Envy or Ivy, compared to the parent GFP variants (Figure 5A). In addition, we performed immunoprecipitation of Bmh2-EGFP, Bmh2-Envy and Bmh2-Ivy using the GFP-nanotrap technology, which employs high affinity nano-bodies derived from llama single chain antibodies [Rothbauer *et al.* 2008]. Our new fusion protein GFP variants were all immunoprecipitated at a similar level to EGFP (Figure 5B), indicating compatibility with the GFP-nanotrap technology.

DISCUSSION

Here we describe two new GFP hybrids we have named Envy and Ivy, which both display optimized characteristics compared to their parent proteins. Both GFP variants are derived from proteins that are codon-optimized for yeast and are incorporated into a convenient and well-established plasmid backbone for the C-terminal tagging of yeast proteins. We also demonstrate that these hybrids remain compatible with commercially available GFP antibody and GFP-nanotrap technology.

We found that Envy exhibited superior brightness compared to all GFP variants tested (Figure 3 and Table 4). This was true at both 30°C and 37°C, suggesting that Envy may have utility beyond studies in yeast. Although Envy was not as photostable as Ivy, its photostability was similar to the enhanced photostability variant GFP γ . Thus, even though Ivy was the most photostable variant tested, the brightness of Envy surpassed Ivy to such an extent that we would recommend Envy as the preferred GFP variant for fusion protein construction.

Envy and Ivy differ at three of their ~230 amino acids (Figure 1). Interestingly, Envy and Ivy differ at residues 69 and 203, whose side chains are located within the beta barrel of GFP. These residues were targeted for rational improvement of GFP during the design of Clover, and found to affect photostability and quantum yield [Lam *et al.*, 2012]. Another difference between Envy and Ivy is at position 206, whose side chain is outside the β barrel [Yang *et al.*, 1996], and does not affect chromophore properties in other GFP variants [Zacharias *et al.*, 2002]. Furthermore, Ivy has a valine immediately following the initial methionine (like its parent protein Clover); complement the *S. cerevisiae* his3 mutation was engineered into Clover so that there would be a more ideal Kozak sequence. Like Clover, Ivy also has an eleven amino acid truncation at the C-terminus relative to Envy. Further studies will be needed to determine which residues lead to the increased photostability and brightness of our mutants.

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EGFP	1	MSKGEELFTGVVPILVELDGDVNGHKFSVS	GE	EGDATY	GKLT	LKFI	CTTGKLPVPWPTL	60
Envy	1	MSKGEELFTGVVPILVELDGDVNGHKFSV R	GE	EGDAT N	GKLT	LKFI	CTTGKLPVPWPTL	60
Ivy	1	MSKGEELFTGVVPILVELDGDVNGHKFSV R	GE	EGDAT N	GKLT	LKFI	CTTGKLPVPWPTL	60
Superfolder	1	MSKGEELFTGVVPILVELDGDVNGHKFSV R	GE	EGDAT N	GKLT	LKFI	CTTGKLPVPWPTL	60
yoClover	1	MSKGEELFTGVVPILVELDGDVNGHKFSV R	GE	EGDAT N	GKLT	LKFI	CTTGKLPVPWPTL	60
GFP γ	1	MSKGEELFTGVVPILVELDGDVNGHKFSVS	GE	EGDATY	GKLT	LKFI	CTTGKLPVPWPTL	60
▲ ▲								
EGFP	61	VTTLTYGVQCF	S	RYPDHMKQHDFFKSAM	PEGYVQERTI	FFKDDG	NYKTRAEVKFEGDTLV	120
Envy	61	VTTL C YGVQCF A	RYPDHMKQHDFFKSAM	PEGYVQERTI	S FKDDG T YKTRAEVKFEGDTLV	120		120
Ivy	61	VTTL C YGV A CF A	RYPDHMKQHDFFKSAM	PEGYVQERTI	S FKDDG T YKTRAEVKFEGDTLV	120		120
Superfolder	61	VTTLTYGVQCF	S	RYPDHMKQHDFFKSAM	PEGYVQERTI	S FKDDG T YKTRAEVKFEGDTLV	120	120
yoClover	61	VTT F GYGV A CF	S	RYPDHMKQHDFFKSAM	PEGYVQERTI	S FKDDG T YKTRAEVKFEGDTLV	120	120
GFP γ	61	VTTL C YGVQCF A	RYPDHMKQHDFFKSAM	PEGYVQERTI	FFKDDG	NYKTRAEVKFEGDTLV	120	120
▲ ▲ ▲ ▲								
EGFP	121	NRIELKGIDFKEDGNILGHKLEYN	YNSHN	VYIMADKQKNGIKVNF	KIRHNIEDG	SVQLAD	180	180
Envy	121	NRIELKGIDFKEDGNILGHKLEYN F	NSHN	VYI T ADKQKNGIK A NFK T RHN V EDG	SVQLAD	180		180
Ivy	121	NRIELKGIDFKEDGNILGHKLEYN F	NSHN	VYI T ADKQKNGIK A NFK T RHN V EDG	SVQLAD	180		180
Superfolder	121	NRIELKGIDFKEDGNILGHKLEYN F	NSHN	VYI T ADKQKNGIK A NFK T RHN V EDG	SVQLAD	180		180
yoClover	121	NRIELKGIDFKEDGNILGHKLEYN F	NSHN	VYI T ADKQKNGIK A NFK T RHN V EDG	SVQLAD	180		180
GFP γ	121	NRIELKGIDFKEDGNILGHKLEYN	YNSHN	VYIMADKQKNGIK A NFK T RHNIEDG	SVQLAD	180		180
▲ ▲ ▲ ▲								
EGFP	181	HYQQNTPIGDGPVLLPDNHYLSTQ	SALS	KDPNEKRDH	MVLEFVTAAGITL	GMDELYK	238	238
Envy	181	HYQQNTPIGDGPVLLPDNHYLSTQ S	V LSKDPNEKRDH	MVLEFVTAAGITL	GMDELYK	238		238
Ivy	181	HYQQNTPIGDGPVLLPDNHYLS H	QSALS	KDPNEKRDH	MVLEFVTA-----	227		227
Superfolder	181	HYQQNTPIGDGPVLLPDNHYLSTQ S	V LSKDPNEKRDH	MVLEFVTAAGITL	GMDELYK	238		238
yoClover	181	HYQQNTPIGDGPVLLPDNHYLS H	QSALS	KDPNEKRDH	MVLEFVTA-----	227		227
GFP γ	181	HYQQNTPIGDGPVLLPDNHYLSTQ	SALS	KDPNEKRDH	MVLEFVTAAGIT	HGMDELYK	238	238
▲ ▲								

Figure 1. Alignment of GFP variants with EGFP

Sequence changes relative to EGFP are noted in bold and indicated by arrowheads. Clover and Ivy contain an additional valine after the initiator methionine. We have omitted this valine in the alignment shown to maintain congruent numbering among the GFP variants. Alignment was produced using ClustalW.

Figure 2. GFP variants Envy and Ivy are in a vector backbone for yeast C-terminal tagging
 (A) Plasmid map showing the GFP variant within the vector for yeast protein C-terminal tagging. The selectable marker on this plasmid is the *S. pombe his5⁺* gene, which complements the *S. cerevisiae his3* mutant. (B) Amplification of the GFP variant within the vector, using indicated primers, will yield the linear DNA fragment shown. The checking primer can be used along with a gene specific primer to confirm proper integration of the GFP variant within the genome. Fwd indicates the position of the vector sequence for the Forward primer. Rev indicates the position of the vector sequence for the Reverse primer. F-Ch indicates the position for the Forward Checking primer.

Figure 3. Brightness levels of GFP variants

Brightness of GFP was calculated from measurements of Bmh2-GFP fluorescence, as described in Materials and Methods. Brightness levels are shown relative to the brightness of Bmh2-EGFP. Strains used to measure brightness levels are: LH1001 (EGFP), LH1002 (SuperfolderGFP), LH10003 (GFP γ), LH1005 (Ivy) and LH10006 (Envy). The number of individual cells measured for each GFP variant (n) is given above each bar. Error bars show the standard error of the mean.

Figure 4. Photostability measurements of GFP variants

Photostability was measured during continuous illumination of cells over the indicated time span. The change in brightness was plotted by measuring the brightness over time and comparing that value to the brightness of the original sample at $t=0$. The brightness of each strain was given as percentage of the initial ($t=0$) brightness measurement. Strains used for brightness measurements are: LH1001 (EGFP), LH1002 (SuperfolderGFP), LH10003 (GFP γ), LH1005 (Ivy) and LH10006 (Envy). The number of individual cells measured for each GFP variant (n) is given above each bar. Error bars show the standard error of the mean.

Figure 5. Compatibility of GFP variants with protein technologies

(A) Top: Immunoblot (IB) probed with JL-8 monoclonal to detect the different GFP variants. Bottom: Immunoblot probed with anti-Pgk1, used as a loading control. Strains used for Western analysis are: LH1001 (EGFP), LH1002 (SuperfolderGFP), LH10003 (GFP γ), LH1005 (Ivy) and LH10006 (Envy). (B) Top: Immunoprecipitation (IP) of GFP variants using the *Camelidae* GFP antibody nanotrap technology. Lysate blots probed with anti-GFP antibody (JL-8) (middle) and anti-Pgk1 antibody (bottom). "Beads" indicates a control immunoprecipitation reaction where agarose beads, instead of agarose beads conjugated with GFP nanobodies, were used to immunoprecipitate EGFP. Strains used for immunoprecipitation are: LH1001 (EGFP), LH1005 (Ivy) and LH10006 (Envy).

Table 1

Plasmids used in this study

Plasmid	Mutations relative to EGFP	Source
pFA6a-link-yoEGFP-SpHis5	T65C S72A V164A I167T L231H [‡]	Lee <i>et. al.</i> , 2013
pFA6a-link-yoClover-SpHis5	S30R Y39N L64F T65G Q69A F99S N105T Y145F M153T V163A I171V T203H	Lee <i>et. al.</i> , 2013
pFA6a-link-yoSuperfolderGFP-SpHis5	S30R Y39N F99S N105T Y145F M153T V163A I171V A206V	Lee <i>et. al.</i> , 2013
pFA6a-link-yoGFPgamma-SpHis5	L64F T65C S72A V163A I167T L231H [‡]	Lee <i>et. al.</i> , 2013
pFA6a-link-yoGFPy-SpHis5	T65C S72A V163A I167T L231H [‡]	This Study
pFA6a-link-Ivy-SpHis5	S30R Y39N T65C Q69A S72A F99S N105T Y145F M153T V163A I167T I171V T203H	This Study
pFA6a-link-Envy-SpHis5	S30R Y39N T65C S72A F99S N105T Y145F M153T V163A I167T I171V A206V	This Study

All mutations are numbered relative to EGFP. Clover and its derivative Ivy have a valine following the initiator methionine. We do not include this valine in the numbering of the mutations so that the amino acid numbering for all GFP variants is congruent.

‡: L231H believed to be a neutral mutation (Shaner *et. al.* 2005)

Table 2*S. cerevisiae* strains used in this study

Strain	Genotype	Source
LH175	MATa <i>ho::hisG lys2 ura3 leu2 his3 trp1 FA</i>	Huang <i>et al.</i> , 2005
LH1001	MATa <i>ho::hisG lys2 ura3 leu2 his3 trp1 FA BMH2-EGFP-his5⁺</i>	This Study
LH1002	MATa <i>ho::hisG lys2 ura3 leu2 his3 trp1 FA BMH2-SuperfolderGFP-his5⁺</i>	This Study
LH1003	MATa <i>ho::hisG lys2 ura3 leu2 his3 trp1 FA BMH2-GFP^γ-his5⁺</i>	This Study
LH1005	MATa <i>ho::hisG lys2 ura3 leu2 his3 trp1 FA BMH2-GFP^{lvy}-his5⁺</i>	This Study
LH1006	MATa <i>ho::hisG lys2 ura3 leu2 his3 trp1 FA BMH2-GFP^{Emv}-his5⁺</i>	This Study

Table 3

Primers used in this study.

Primer name	Sequence (5'→3')
OLH1501	GGCCAACCTTGGTGACTACGTTATGTTATGGCGTACAATG
OLH1502	CGTAGTCACCAAAGTTGGCCATGGTACTG
OCS1	CAACAAGCTCCAGCTGAACAACTCAAGGTGAACCAACCAAGGTGACGGTGCTGGTTTA
OCS2	GGAGTGGTAAATCTTCATTTCCCTTGTATTCTCAGCGCTCTCGATGAATTCGAGCTCG
OCS3	CTACATTAGTAACAACATTATGTTACGGAGTTCAATGCTTTGCTAGATACCCAG
OCS4	TAATGTTGTTACTAATGTAGGCCATGGAAC
OCS5	GTATAAAAGCGAACTTTAAGACAAGACATAACG
OCS6	CTTAAAGTTCGCTTTTATACCATTCCTTTTG
OCS7	GGCCAACGTTAGTTACAACCTTTATGTTATGGTGTAGCTTGCTTTGCTAGGTACCCAG
OCS8	AGTTGTAACCTAACGTTGGCCATGGTACAGG
OCS9	GTATAAAAGCCAACCTTTAAGACAAGACATAACG
OCS10	CTTAAAGTTGGCTTTTATACCATTCCTTTTG

Table 4

Relative brightness of GFP variants at 30°C and 37°C

Strain name	Relevant genotype	Brightness at 30°C (Mean ± S.E.)	Brightness at 37°C (Mean ± S.E.)	p-value at 30°C	p-value at 37°C
LH1001	<i>BMH2-EGFP</i>	1.00 ± 0.04	1.00 ± 0.03	-	-
LH1002	<i>BMH2-SuperfolderGFP</i>	1.03 ± 0.04	1.60 ± 0.07	5.48×10^{-01}	5.44×10^{-14}
LH1003	<i>BMH2-GFPγ</i>	1.22 ± 0.05	2.09 ± 0.09	1.01×10^{-03}	2.91×10^{-20}
LH1005	<i>BMH2-Ivy</i>	1.08 ± 0.06	1.31 ± 0.05	2.52×10^{-01}	7.65×10^{-07}
LH1006	<i>BMH2-Envy</i>	2.18 ± 0.09	3.97 ± 0.11	3.91×10^{-21}	1.83×10^{-66}

Brightness is relative to EGFP; p-values are for comparing the GFP variant with EGFP.

Table 5

Photostability of GFP variants

Strain name	Relevant genotype	% Initial Brightness 60 sec. (Mean $\pm$ S.E.)	% Initial Brightness 120 sec. (Mean $\pm$ S.E.)	p-value compared to EGFP at $t = 60$ sec.	p-value compared to Ivy at $t = 120$ sec.
LH1001	<i>BMH2-EGFP</i>	7.6 ± 1.1	<i>n/a</i>	<i>n/a</i>	<i>n/a</i>
LH1002	<i>BMH2-SuperfolderGFP</i>	10.9 ± 0.8	<i>n/a</i>	1.5×10^{-2}	<i>n/a</i>
LH1003	<i>BMH2-GFPγ</i>	59.0 ± 1.0	38.8 ± 1.3	3.3×10^{-43}	1.4×10^{-8}
LH1005	<i>BMH2-Ivy</i>	74.3 ± 1.0	51.8 ± 1.5	1.7×10^{-49}	<i>n/a</i>
LH1006	<i>BMH2-Envy</i>	59.9 ± 0.9	39.3 ± 1.2	2.6×10^{-49}	8.9×10^{-9}