Yeast
Yeast 2004; **21**: 661–670.
Published online in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/yea.1130

Yeast Functional Analysis Report

Optimized cassettes for fluorescent protein tagging in *Saccharomyces cerevisiae*

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Received: 15 November 2003
Accepted: 8 March 2004

Abstract

Green fluorescent protein (GFP) has become an increasingly popular protein tag for determining protein localization and abundance. With the availability of GFP variants with altered fluorescence spectra, as well as GFP homologues from other organisms, multi-colour fluorescence with protein tags is now possible, as is measuring protein interactions using fluorescence resonance energy transfer (FRET). We have created a set of yeast tagging vectors containing codon-optimized variants of GFP, CFP (cyan), YFP (yellow), and Sapphire (a UV-excitable GFP). These codon-optimized tags are twice as detectable as unoptimized tags. We have also created a tagging vector containing the monomeric DsRed construct tdimer2, which is up to 15-fold more detectable than tags currently in use. These tags significantly improve the detection limits for live-cell fluorescence imaging in yeast, and provide sufficient distinguishable fluorophores for four-colour imaging. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords: green fluorescent protein; red fluorescent protein; DsRed; multicolour fluorescence

Introduction

Functional analysis of *Saccharomyces cerevisiae* genes has been greatly aided by the ease with which genes can be deleted or tagged by homologous recombination into the genome. Cassettes for tagging genes with a wide range of sequences have been developed, including epitope tags for immunochemistry, protein purification tags and green fluorescent protein (GFP) variants for microscopy. Because the tags are introduced by directed recombination into the genome, these cassettes allow modification of the targeted protein in its native context without perturbing its native regulation. These tags have become essential tools for the study of yeast genes. Fluorescent protein fusions have been particularly useful, as they allow fluorescence microscopy of live cells with a minimum of perturbation. A number of plasmids have been specifically designed for this purpose, encoding GFP, YFP or CFP with a variety of selectable markers (Hailey *et al*., 2002; Reid *et al*., 2002; Wach *et al*., 1997). Furthermore, fluorescent protein fusions can be used to detect the interaction of two proteins by fluorescence resonance energy transfer (FRET).

Because these tagging cassettes can be fused to proteins under the expression of their native promoter, the level of the expressed protein is often quite low, making it difficult to detect the fusion protein. To improve the expression of fluorescent protein fusions, we have constructed a set of four different wavelength GFP variants which have been optimized to use the most abundant codons in yeast. These plasmids are derivatives of pFA6a (Wach *et al*., 1997) and encode a codon-optimized GFP variant (Cormack *et al*., 1997) and a selectable marker (*Schizosaccharomyces pombe HIS5, C. albicans URA3*, or KanR). The GFP variants encoded in these plasmids are codon-optimized GFP, CFP and Sapphire (Tsien, 1998) and the improved YFPs Venus (Nagai *et al*., 2002) and Citrine. We find that these tags are up to twice as bright as unoptimized tags. To further improve the utility of these proteins, we have generated true monomeric CFP and YFP constructs which incorporate the

A206R mutation to block dimerization (Zacharias *et al*., 2002), and CFP and YFP constructs fused to the 3HA and 13Myc epitope tags (Evan *et al*., 1985; Field *et al*., 1988; Longtine *et al*., 1998) to allow use of both fluorescence and immunochemical approaches. Additionally, we have constructed a tagging cassette for generating fusions to the monomeric DsRed variant tdimer2 (Campbell *et al*., 2002). This fluorophore is up to 15-fold brighter than unoptimized YFP. Together, these cassettes provide sufficient fluorophores for simultaneous four-colour imaging, as well as multiple potential FRET pairs.

Materials and methods

Strains and media

Escherichia coli host strains were XL-10 gold and DH5α. Yeast strain JYL69 (*MAT***a** *ura3-1 ADE2+ his3-11,15 leu2-3,112 trp1-1 can1-100*; isogenic to W303) was used for all experiments. Standard yeast media were used for selection of transformants (Sherman, 2002). For fluorescence measurements, yeast were grown in synthetic complete or the appropriate drop-out media made using low-fluorescence yeast nitrogen base (yeast nitrogen base without riboflavin and folic acid; 5 g/l $(NH_4)_2SO_4$, 1 g/l KH_2PO_4, 0.5 g/l $MgSO_4$, 0.1 g/l NaCl, 0.1 g/l Ca_2Cl, 0.5 mg/l H_3BO_4, 0.04 mg/l $CuSO_4$, 0.1 mg/l KI, 0.2 mg/l $FeCl_3$, 0.4 mg/l $MnSO_4$, 0.2 mg/l Na_2MoO_4, 0.4 mg/l $ZnSO_4$, 2 µg/l biotin, 0.4 mg/l calcium pantothenate, 2 mg/l inositol, 0.4 mg/l niacin, 0.2 mg/l PABA, 0.4 mg/l pyridoxine HCl, 0.4 mg/l thiamine). This medium has negligible autofluorescence (within 10% of water). We have found that commercial yeast nitrogen base, even without vitamins, is often highly fluorescent and so we prepare our own from the above components, purchased from Sigma.

Construction of optimized tagging vectors

All primers used are described in Table 1. Codon-optimized green fluorescent protein (yEGFP1; Cormack *et al*., 1997) was amplified by PCR using primers F2 and R2, adding *Pac*I and *Asc*I sites, respectively, and cloned into plasmid pDH5 (Hailey *et al*., 2002), replacing YFP to give pFA6a–yEGFP1–*SpHIS5* (Figure 1). The selectable marker, *Sz. pombe HIS5* (*SpHIS5*), complements *S. cerevisiae HIS3*. The yEGFP cloned into this plasmid contains a point mutation converting Met 233 to Ile. This mutation is present in all GFP variants subsequently derived from this plasmid. As this residue is disordered and not observed in the GFP crystal structure, we expected it to have little effect on the fluorescence properties of GFP. We verified this by reverting this mutation in our yEVenus construct and found that it had no effect on fluorescence. We have therefore left this mutation in all the GFP derivatives described here.

An optimized linker was introduced into the yEGFP construct by phosphorylating primers L1 and L2, annealing them together, and ligating them into pFA6a–yEGFP1–*SpHis5* cut with *Bam*HI and *Pac*I, yielding pFA6a–link–yEGFP1–*SpHIS5*. GFP variants were constructed by site-directed mutagenesis of yEGFP1 using the Quikchange multi and Quikchange XL kits (Stratagene). In all cases, the replacement codons were chosen to be frequently used codons in yeast genes. Following mutagenesis, the GFP variant sequence was confirmed by sequencing and then subcloned into the unmutagenized pFA6a vector using the *Pac*I and *Asc*I sites. The final GFP mutants contain the following amino acid changes relative to: wild-type GFP — yEGFP1, F64L, S65T; yEVenus — F46L, F64L, S65G, S72A, M153T, V163A, S175G; yECitrine — S65G, V68L, Q69M, S72A, T203Y; yECFP — F64L, S65T, Y66W, N146I, M153T, V163A; yESapphire — S72A, Y145F, T203I.

yEYFP, used as an intermediate in the construction of yEVenus and yECitrine, was constructed from yEGFP1 by introducing mutations S65G, V68L, S72A and T203Y with the Quikchange multi-kit, using pFA6a–yEGFP1–*SpHIS5* as template and mutagenic primers M2 and M1. yEVenus was constructed from yEYFP using the Quikchange multi-kit, with plasmid pDH5–yEYFP as template and mutagenic primers M3, M4 and M5, adding mutations F46L, M153T, V163A and S175G. yECitrine was made using the Quikchange XL kit, with pDH5–yEYFP as template and mutagenic primers M8 and M12, adding mutation Q69M.

To make yECFP, mutations Y66W, N146I and V163A were introduced with the Quikchange multi-kit, using yEGFP1 as the template and mutagenic primers M4, M6 and M7. Mutation M153T was then introduced to the resulting plasmid

Table 1. Oligonucleotides

Oligonucleotide	Sequence (5′ — 3′)
M1	caaccattacttatcc**TAT**caatctgccttatcc
M2	ttagtcactacttta**GGT**tatggt**TTG**caatgtttt**GCT**agatacccagatcat
M3	cggtaaattgaccttaaaa**ttG**atttgtactactggt
M4	caatgtttacatc**aCT**gctgacaaacaaaagaatggtatcaaa**gCt**aacttcaaaattaga
M5	caacattgaagatggt**GGt**gttcaattagctgacc
M6	ccttagtcactactttaact**tGG**ggtgttcaatgtttttc
M7	cattttaggtcacaaattggaatac**aTT**ataactctca
M8	ttaggttatggtttg**ATG**tgttttgctagatac
M9	cttagtcactact**ttTTCT**tatggtgttcaatgtttt**GCT**agatacccagatcat
M10	aaattggaatacaac**TTT**aactctcacaatgtt
M11	aaccattacttatcc**ATT**caatctgccttatcc
M12	gtatctagcaaaaca**CAT**caaaccataacctaa
M13	ttaggttatggtttg**ATG**tgttttgctagatac
M14	atggatgaattgtacaaa*CCCGGG*GGCTCC*GAATTC*ggcgcgccacttctaaat
M15	atttagaagtggcgcgcc*GAATTC*GGAGCCCCCC*GGG*tttgtacaattcatccat
M16	gacaaccattacttatccactcaatct**AGA**ttatccaaagatccaaacgaaaagaga
M17	tctcttttcgtttggatctttggataa**TCT**agattgagtggataagtaatggttgtc
M18	gacaaccattacttatcctatcaatct**AGA**ttatccaaagatccaaacgaaaagaga
M19	tctcttttcgtttggatctttggataa**TCT**agattgataggataagtaatggttgtc
L1	Gatcggtgacggtgctggtttaat
L2	Taaaccagcaccgtcacc
F1	gatc*ttaattaa*Catggtggcctcctccgag
R1	Gatc*ggcgcgcc*ctacaggaacaggtggtg
F2	Gcatc*ttaattaa*catgtctaaaggtgaagaattattc
R2	Gcatc*ggcgcgcc*ttatttgtacaattcatccatacc
F3	(Gene-specific sequence)-ggtcgacggatccccggg
R3	(Gene-specific sequence)-tcgatgaattcgagctcg
F4	(Gene-specific sequence)-ttaattaacatgtctaaaggtg
F5	(Gene-specific sequence)-ggtgacggtgctggttta

Lower case, original sequence; capitals, introduced or mutated bases; boldface, mutated codons; italics, restriction sites.

with the Quikchange XL kit, using primers M4 and M13. yESapphire was made by using the Quikchange multi kit with yEGFP as template and mutagenic primers M9, M10 and M11 to create mutations S72A, Y145F and T203I.

The G418 resistance marker (KanR) was introduced into these plasmids by subcloning the *Bgl*II–*Eco*RI fragment of pDH3 (Hailey *et al*., 2002) into the GFP variant plasmids. *Candida albicans URA3* (*CaURA3*) was introduced by subcloning the *Bgl*II–*Sac*I fragment of pAG60 (Goldstein *et al*., 1999) into the GFP variant plasmids.

To construct pFA6a–link–tdimer2–*SpHis5*, tdimer2 (Campbell *et al*., 2002) was amplified by PCR with primers F1 and R1, adding *Pac*I and *Asc*I sites, and cloned into pFA6a–link–yEVenus-*SpHIS5*.

To fuse the epitope tags 3HA and 13Myc onto the fluorescent tags in our tagging cassettes, we first created novel *Xma*I and *Eco*RI restriction sites and removed the stop codon from yECFP and yECitrine by site-directed mutagenesis with the Quikchange XL kit (Stratagene) and mutagenic primers M14 and M15. The 3HA tag was then subcloned into pKT102 and pKT140, using the new *Xma*I site and existing *Asc*I site. The 13Myc tag was subcloned into pKT102 and pKT140 using the new *Xma*I and *Eco*RI sites.

The A206R mutation in yECitrine and yECFP was introduced using the Quikchange XL kit (Stratagene) and mutagenic primers M16 and M17 (yECFP) and M18 and M19 (yECitrine). The resulting yEmCit and yEmCFP constructs were then subcloned back into their unmutagenized vectors using the *Pac*I and *Asc*I restriction sites.

Gene tagging

Plasmids (Table 2) were amplified using the long-template PCR system (Roche) following the

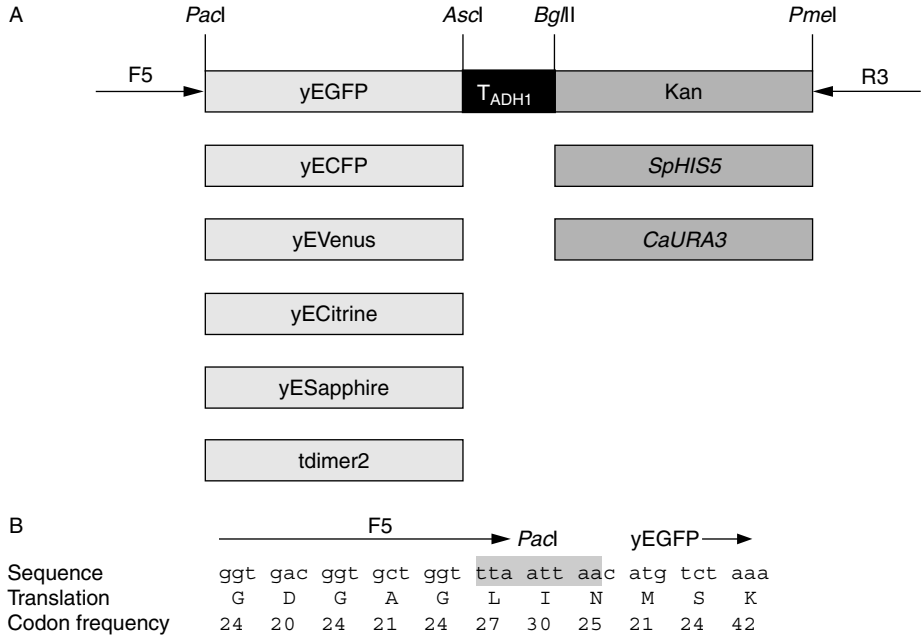

Figure 1. Overview of the new tagging constructs. (A) Structure of the tagging constructs. The organization is similar to other common tagging constructs, with a forward PCR primer (F5) followed by the fluorophore to be fused to the gene of interest, the *S. cerevisiae ADH1* terminator and a selectable marker. The sequences used to amplify these cassettes are 5′-(gene-specific sequence)-GGTGACGGTGCTGGTTTA-3′ (F5) and 5′-(gene-specific sequence)-TCGATGAATTCGAGCTCG-3′ (R3). Restriction sites at the boundaries between elements are indicated. Sequences are not drawn to scale. (B) Sequence of the linker between the target protein and yEGFP. The translation and frequency of each codon in the yeast genome (per 1000) are given. For reference, the most frequent codon in the yeast genome is GAA (glutamate), with a frequency of 46/1000. The least frequent non-stop codon is CGG (arginine), with a frequency of 1.7/1000. Codon frequencies are from **http://www.kazusa.or.jp/codon/** (Nakamura *et al.*, 2000)

manufacturer's instructions, with an annealing temperature of 65 °C and an extension time of 140 s. The forward primer consisted of the 40 3′ nucleotides of the gene to be tagged (excluding the stop codon) fused to F5; the reverse primer consisted of the reverse complement of the 40 nucleotides 3′ of the stop codon fused to R3. Tagging of the targeted gene was confirmed by colony PCR to confirm the presence of both integration junctions and the absence of the unmodified gene (Petracek and Longtine, 2002).

Fluorometry and microscopy

The fluorescence of yeast cultures was measured in black 96-well plates with a Spectramax Gemini XS. Excitation and emission wavelengths were chosen to maximize the ratio of fluorescence intensities of the tagged strain to the untagged strain. The following excitation/emission pairs (in nm) were used: Sapphire, 399/508; GFP, 476/512; YFP and variants, 502/532; tdimer2, 552/579. In all cases the 'autocutoff' feature of the instrument was used. To correct for variations in cell number, these fluorescence intensities were then normalized by the OD_{660} of the culture measured in transparent 96-well plates with a Spectramax Plus 384.

Microscopy was performed on a Zeiss Axioscop 2 with a Plan-NeoFluar 100×/1.3 NA oil immersion objective. Images were recorded on a Zeiss Axiocam MRm with 2×2 binning. The following filter sets were used: YFP, Chroma No. 41 028, HQ500/20 exciter, Q515lp beamsplitter, HQ535/30 emitter; GFP, Zeiss No. 38, BP470/40 exciter, FT495 beamsplitter, BP525/50 emitter; tdimer2, Zeiss No. 43, BP545/20 exciter, FT570 beamsplitter, BP605/70 emitter. Cells were pelleted and resuspended in SC without glucose and immobilized on concanavalin A-coated coverslips for imaging. Cdc11p-tagged strains were arrested

Table 2. Plasmids generated in this study

Plasmid	Full name
pKT128	pFA6a–link–yEGFP–*SpHIS5*
pKT127	pFA6a–link–yEGFP–Kan
pKT209	pFA6a–link–yEGFP–*CaURA3*
pKT149	pFA6a–link–ySapphire–*SpHIS5*
pKT150	pFA6a–link–ySapphire–Kan
pKT146	pFA6a–link–tdimer2–*SpHIS5*
pKT178	pFA6a–link–tdimer2–Kan
pKT176	pFA6a–link–tdimer2–*CaURA3*
pKT101	pFA6a–link–yECFP–*SpHIS5*
pKT102	pFA6a–link–yECFP–Kan
pKT174	pFA6a–link–yECFP–*CaURA3*
pKT90	pFA6a–link–yEVenus–*SpHIS5*
pKT103	pFA6a–link–yEVenus–Kan
pKT139	pFA6a–link–yECitrine–*SpHIS5*
pKT140	pFA6a–link–yECitrine–Kan
pKT175	pFA6a–link–yECitrine–*CaURA3*
pKT210	pFA6a–link–yEmCFP–*SpHIS5*
pKT212	pFA6a–link–yEmCFP–*CaURA3*
pKT211	pFA6a–link–yEmCitrine–*SpHIS5*
pKT220	pFA6a–link–yECitrine–3HA–KANr
pKT221	pFA6a–link–yECFP–3HA–KANr
pKT232	pFA6a–link–yECFP–13Myc–KANr
pKT233	pFA6a–link–yECitrine–13Myc–KANr
pKT239	pFA6a–link–yECitrine–3HA–*SpHIS5*
pKT240	pFA6a–link–yECitrine–13Myc–*SpHIS5*

with 15 μg/ml nocodazole prior to imaging. Exposure times were 1 s for Nup49p-tagged strains and 500 ms for Cdc11p-tagged strains. Images for Figure 3 were acquired with a Orca II ER camera through an alphaFluor 100×/1.45 NA objective on a Zeiss Axiovert 200M microscope. The filter sets were those described above. The excitation illumination was attenuated by 75% with a neutral density filter and the camera was operated in high precision mode with 2 × 2 binning.

Distribution of plasmids

All plasmids are available from EUROSCARF (**http://www.uni-frankfurt.de/fb15/mikro/euroscarf/index.html**). The GFP variants (ySapphire, yECFP, yEGFP, yEVenus and yECitrine) are freely available for non-commercial use; commercial users must obtain a licence from Vertex pharmaceuticals (Cambridge, MA). All users of the tdimer2 plasmids must sign an MTA with Dr Roger Tsien (UCSD) before obtaining the plasmid.

Results and discussion

Determinants of fluorescent intensity

Fusion of a DNA sequence to the 3′ end of an open reading frame in its native genomic context does not affect the promoter or upstream regulatory regions. We therefore expect that C-terminal fluorescent protein fusions will be transcribed at levels comparable to the untagged protein. The brightness of the fusion depends primarily on three properties of the fluorescent protein: its intrinsic brightness, folding efficiency, and translation efficiency. Increasing any one of these will increase the brightness of the fusion protein. Under long exposures in a fluorescence microscope, the photobleaching rate of the protein will also be important.

The intrinsic brightness of a fluorescent protein (FP) is the product of the fluorescent quantum yield and the extinction coefficient, and is affected only by the sequence of the FP and by the surrounding solution (as many FPs are quenched by low pH or high chloride). The folding efficiency is also primarily determined by the sequence of the FP and the surrounding environment (temperature and pH) and possibly by interference between the FP and the protein to which it is fused. Translation efficiency is affected primarily by the codon usage of the FP, and altering the codon usage of FPs to conform more closely to the preferred codons in the expression host has been shown to improve expression of the FP (Cormack *et al*., 1997; Crameri *et al*., 1996). As inefficient translation or inefficient folding reduce expression compared to that of the untagged protein, we expect that improvements in brightness from codon optimization result from restoring expression of the fusion protein towards that of the untagged protein.

For most purposes, however, what matters is not the absolute brightness of the fluorescent protein fusion, but its brightness relative to the background fluorescence (autofluorescence) of yeast. Because modern imaging systems are sensitive enough to detect the autofluorescence of yeast easily, this sets the lower limit for FP detection. Thus, it can be advantageous to use a protein that fluoresces in a region of the spectrum with low yeast autofluorescence, even if this protein is less bright than others. In this paper, we calculate the detectability of a fluorescent tag as the ratio intensity of the fluorescent tag : intensity of the yeast autofluorescence, giving a signal-to-background ratio (SBR).

Construction of tagging plasmids

We set out to build an improved set of fluorescent tagging plasmids for use in yeast. We first constructed a tagging vector (pFA6a–yEGFP1–*SpHIS5*) containing the yeast codon-optimized GFP, yEGFP1, which has previously been shown to be substantially brighter than unoptimized GFP when expressed in yeast (Cormack *et al*., 1997). The remainder of this plasmid is identical to pFA6a–GFP–HIS3MX6 (Wach *et al*., 1997). We then introduced mutations into yEGFP1 to construct the improved YFP, Venus (Nagai *et al*., 2002). We expected this yEVenus construct to be brighter than unoptimized Venus due to improved expression resulting from codon optimization.

We compared the fluorescence of our yEVenus construct with the unoptimized Venus by generating fusions of both fluorescent tags to the highly abundant yeast protein Cdc19p (data not shown). Surprisingly, the yEVenus fusion was not significantly brighter than the unoptimized Venus fusion. Examination of the sequence of the resulting fusion construct revealed that the forward primer used to amplify the pFA6a vector, which forms a linker between the tagged protein and the fluorophore (F3; Table 1) included two infrequently used arginine codons (CGA and CGG), which occur in the yeast genome at a frequency of only 0.3% and 0.2% (Nakamura *et al*., 2000). Use of a primer (F4) which did not include these poor codons resulted in a fusion construct twice as bright as the unoptimized Venus (data not shown).

We therefore added an improved linker to our tagging plasmid to give pFA6a–link–yEVenus–*SpHIS5*. This linker must fulfil three functions: it is the primer binding site for PCR amplification of the tagging cassette, and so should be GC-rich to give a high T_m; it forms a linker between the protein and the tag and so should be flexible; and it should be codon-optimized to give high expression. The resulting linker (primer F5; Figure 1) fulfils all of these requirements: it encodes the Gly-rich sequence GDGAGL, it has a T_m of 60 °C, and all codons in it occur frequently in yeast. Tagging of Cdc19p with this new construct gave a strain that was twice as bright as the unoptimized Venus, as expected.

Yeast codon-optimized versions of CFP, Citrine, and Sapphire were then constructed in the vector by site-directed mutagenesis of yEGFP (Figure 1). Citrine is an improved version of YFP that has increased folding efficiency at high temperature, increased resistance to pH and Cl^-, and increased photostability (Griesbeck *et al*., 2001; Miyawaki *et al*., 1999). Sapphire (also called H9-40) is a GFP variant that is maximally excited at 399 nm and emits at 511 nm (Cubitt *et al*., 1999). It is spectrally distinct from the CFP and YFP variants, and is a potential FRET donor to DsRed (Mizuno *et al*., 2001). Additionally, we cloned the tdimer2 variant of DsRed (Campbell *et al*., 2002) into the pFA6a-link plasmid. This protein is a tandem dimer of a dimeric DsRed variant, rendering it a functional monomer. We chose to use tdimer2 because it is substantially brighter than the true monomer, mRFP1. Although it is not yeast codon-optimized, tdimer2 has two advantages as a yeast tag: it is spectrally distinguishable from Sapphire, CFP and YFP, and it fluoresces in the red, where yeast autofluorescence is low.

To further optimize the yECFP and yECitrine tags we generated monomeric versions of both proteins (known as yEmCFP and yEmCitrine) by introducing the A206R mutation (Zacharias *et al*., 2002). GFP variants normally dimerize with a dissociation constant of ~100 μM, which can potentially confound studies of interacting proteins tagged with GFP variants. Introduction of the A206R mutation increases the dissociation constant by about 1000-fold to the point where association is nearly undetectable.

One additional problem when using GFP variants to follow multiple proteins is that because of the high sequence identity of the different variants, an anti-GFP antibody will recognize all GFP variants with equal affinity, precluding the use of GFP antibodies to discriminate between the tagged proteins. To overcome this problem, we have generated tagging vectors containing yECFP and yECitrine with the 3HA and 13Myc tags fused to the C-terminus of the fluorescent protein. The availability of distinct epitope tags for each variant allows the two variants to followed independently (Evan *et al*., 1985; Field *et al*., 1988; Longtine *et al*., 1998) by use of the appropriate antibody.

Testing of new tags

To compare the suitability of these fluorescent tags for use in yeast, we first fused each tag (except for yECFP) to Cdc19p and Tdh3p. We chose not to

study yECFP, as CFP is known to be substantially less bright than YFP and GFP. We also tagged these genes with YFP (from plasmid pDH5; Hailey *et al*., 2002) for comparison to an unoptimized construct. Cdc19p and Tdh3p were chosen because they are highly abundant. Fluorescence of each strain was measured in a 96-well plate reader (Figure 2, Table 3). Because exposure times in this experiment are short and illumination intensities are low, these experiments are not expected to be significantly influenced by photobleaching, and therefore test the utility of these reporters for applications such as plate reader measurements or fluorescence-activated cell sorting.

The fluorescence of each strain was corrected for the number of cells as measured by OD_{660}, and a signal-to-background ratio (SBR) was then calculated by dividing the fluorescence of each strain by the fluorescence of the untagged parent strain under the same conditions. To compare SBRs of tagged Cdc19p and Tdh3p, despite their different expression levels, the SBRs were normalized by the SBR of the YFP-tagged strains. This gives a measure of the increase in detectability over

Table 3. Fluorescence of tagged yeast strains in plate reader

Sample	Average fluorescence	Standard deviation
YFP fluorescence measurements		
TDH3–YFP	856.1	90.3
TDH3–yEVenusL	1690.1	247.8
TDH3–yECitrineL	1735.1	117.7
CDC19–YFP	322.7	26.8
CDC19–yEVenusL	717.5	48.9
CDC19–yECitrineL	661.7	61.7
JYL69 autofluorescence	29.3	5.4
GFP fluorescence measurements		
TDH3–yEGFPL	3429.9	1360.1
CDC19–yEGFPL	1326.5	123.7
JYL69 autofluorescence	107.7	70.3
Sapphire fluorescence measurements		
TDH3–yESapphireL	2318.3	192.5
CDC19–yESapphireL	590.0	73.1
JYL69 autofluorescence	171.0	18.6
Tdimer2 fluorescence measurements		
TDH3–tdimer2	499.6	77.0
CDC19–tdimer2	318.2	55.1
JYL69 autofluorescence	2.1	0.2

Each strain shown was measured in the plate reader as described in the text. The data shown are the average and standard deviation of at least two measurements on separate days.

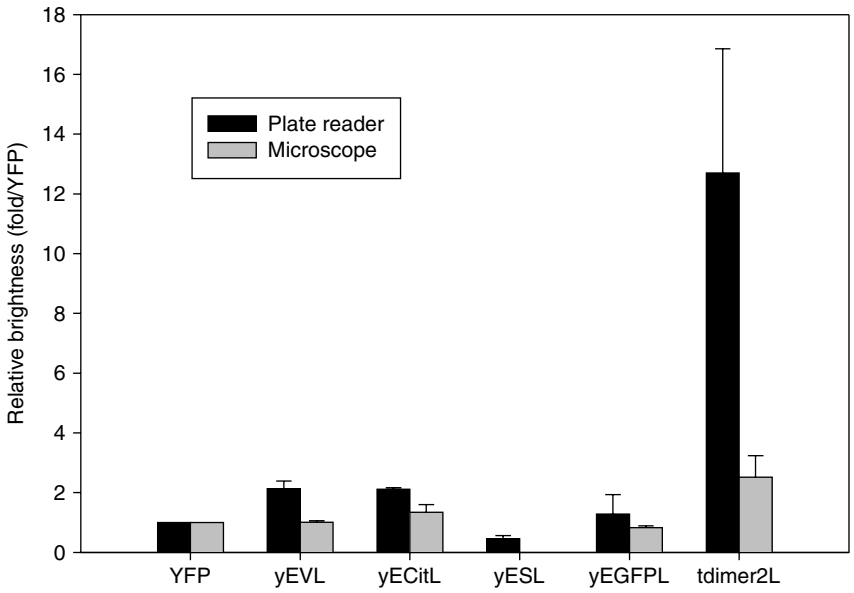

Figure 2. Relative detectability of different fluorophores in yeast. Yeast strains with Tdh3p and Cdc19p (plate reader) or Cdc11p and Nup49p (microscope) were tagged with the fluorophores shown. A signal : background ratio for each sample was calculated by dividing the fluorescence of each strain by the fluorescence of the untagged parent strain under the same conditions. The signal : background ratio for each strain was then divided by that of the corresponding YFP-tagged strain to give the relative brightness, which measures the increase in detectability over unoptimized YFP. Each strain was measured at least twice on separate days, and the data shown are the average and standard deviation of all measurements. yEVL, yeast-enhanced Venus with optimized linker; yECitL, Citrine; yESL, Sapphire

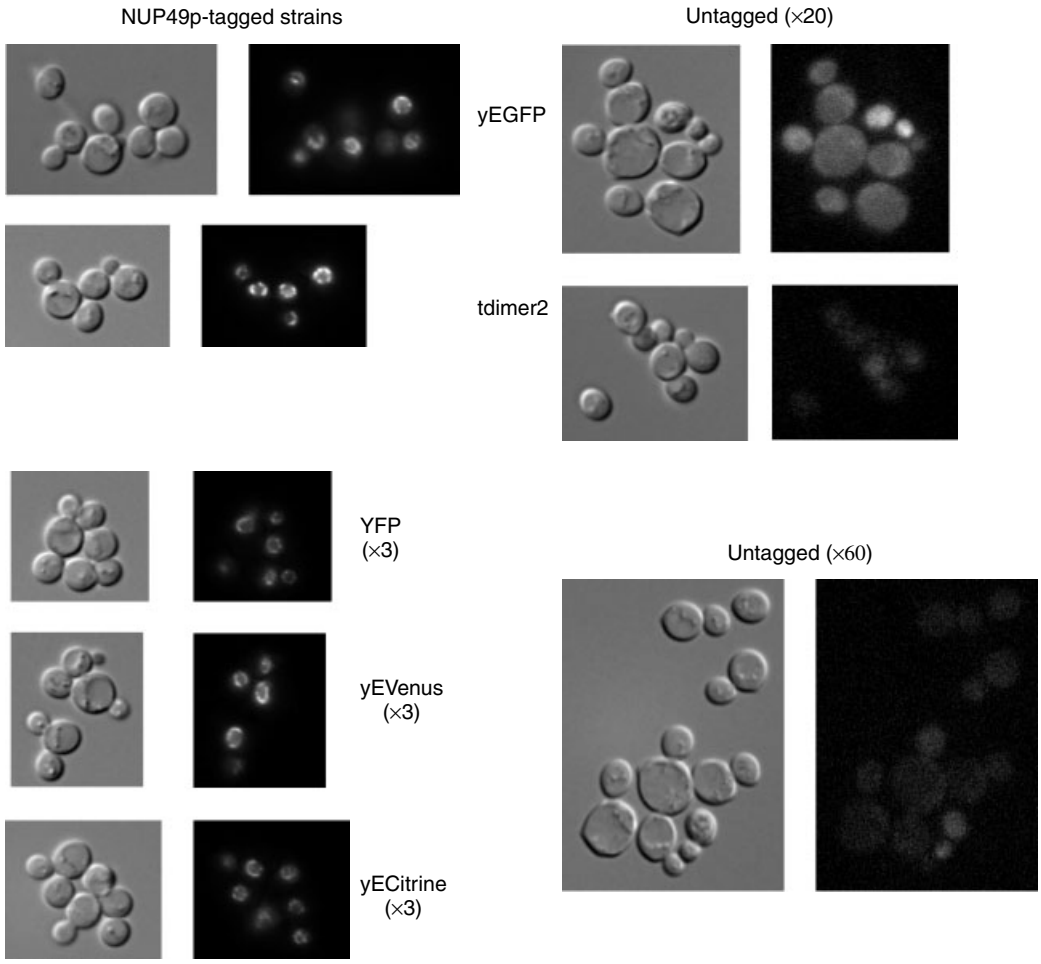

Figure 3. Nup49p-tagged with optimized fluorescent proteins. DIC (left) and fluorescence (right) images of the indicated strains acquired as described in Materials and methods. After acquisition, the images were background-subtracted and scaled identically. Untagged images were acquired on the parent strain using identical settings. The untagged fluorescence images are shown 20-fold brighter than the tagged images. The YFP images (both tagged and untagged) have been scaled up by a factor of three to give the indicated increases in brightness

unoptimized YFP, independent of expression level. The increase in detectability for each strain was measured twice on separate days, and the data shown are the average of both measurements (Table 3).

We found that the results were essentially identical between the Tdh3p and Cdc19p tagged strains, and these data are averaged together in Figure 2. yEVenus and yECitrine have comparable brightness, and are about twice as detectable as unoptimized YFP. yEGFP is slightly less detectable that yEVenus or yECitrine, but is roughly comparable. yESapphire is significantly less detectable than the other tags (~10-fold less than YFP), due to the increased fluorescence of yeast when excited at short wavelengths (Table 3). The tdimer2 tag, although less bright than the others, is much more detectable (~15-fold more so than YFP) because of the very low yeast autofluorescence at long wavelengths. This measurement of yeast autofluorescence is likely to be an overestimate as it is at the limit of detection of the plate reader.

We then tested the suitability of these tags in microscopy applications by fusing each to the low-abundance proteins Nup49p (a nuclear pore component) and Cdc11p (a septin). We chose to use these low-abundance proteins because they provide a more stringent test of the new tags than the

high-abundance proteins used for measurements on the plate reader. Low-abundance proteins require long exposure times (~1 s) to get sufficient signal on the microscope, which can lead to significant photobleaching of the fluorescent protein. This measurement thus tests both the absolute brightness of the tags and their photostability.

We omitted yESapphire from the comparison because of its relative dimness. Images of each tagged strain and the untagged parent were obtained as described in Materials and methods. Representative images are shown in Figure 3. A threshold was then chosen for each set of images to select the tagged protein (or the entire cell for the untagged control). Pixels above this threshold were sorted by intensity, and the intensity of each strain is reported as the minimum intensity that includes 90% of the pixels. For comparison, the signal : background ratio was again normalized to YFP (Figure 2). The results for these measurements are generally similar to the results obtained in the plate reader, with the exception that yECitrine is brighter than yEVenus, especially at longer exposure times, presumably due to its greater photostability.

Tdimer2 performs substantially worse in the microscopy assay than in the plate reader assay. This appears to be because yeast autofluorescence at the tdimer2 emission wavelength is larger when measured on the microscope than in the plate reader (cf. Tables 3 and 4), probably due to the larger bandpasses used for excitation and emission on the microscope. Consistent with this, examination of the autofluorescence spectrum of yeast excited at 552 nm reveals increased fluorescence between 579 nm (the emission wavelength measured in the plate reader) and 570 nm (the short-wavelength cut-off of the emission filter of the microscope). Use of a filter set with a red-shifted emission may improve the signal : background ratio when imaging tdimer2 constructs.

To examine the suitability of these tags for simultaneous four-colour imaging, we mixed cells expressing TDH3 tagged with yESapphire, yECFP, yECitrine, and tdimer2 with untagged yeast. Images of the resulting mixture were acquired as described in Materials and methods (Figure 4). These images were then normalized by the exposure time, and cross-talk between the channels was measured by linear regression of the pixel intensities in the channel corresponding to the tagged fluorophore against the intensities of those pixels in the other channels. The resulting cross-talk values (in %) are shown in Table 5.

Table 4. Fluorescence of tagged yeast strains in microscope

A.

Sample	Average fluorescence	Standard deviation
YFP fluorescence measurements		
NUP49–YFP	481	72
NUP49–yEVenusL	536	6
NUP49–yECitrineL	661	99
JYL69 autofluorescence	46	5
GFP fluorescence measurements		
NUP49–yEGFPL	1689	72
JYL69 autofluorescence	207	56
Tdimer2 fluorescence measurements		
NUP49–tdimer2	1934	177
JYL69 autofluorescence	69	10

B.

Sample	Average fluorescence	Standard deviation
YFP fluorescence measurements		
CDC11–YFP	705	206
CDC11–yEVenusL	912	340
CDC11–yECitrineL	740	436
JYL69 autofluorescence	32	20
GFP fluorescence measurements		
CDC11–yEGFPL	2801	422
JYL69 autofluorescence	114	18
Tdimer2 fluorescence measurements		
CDC11–tdimer2	3747	29
JYL69 autofluorescence	46	10

Each strain shown was imaged on a Zeiss Axioscop and quantitated as described in the text. The data shown are the average and standard deviation of at least two measurements on separate days. Because these are raw intensity measurements, they are subject to fluctuations from changes in the lamp brightness and alignment from one day to the next. Since these errors are common to both the tagged and untagged strain measurements, they cancel when the data are presented as ratios, as in Figure 2. Because of this, the relative brightness reported here differs from the detectability reported in Figure 2.

In summary, we have generated a set of tagging plasmids containing codon-optimized GFP variants and a monomeric DsRed construct. These plasmids provide fluorophores suitable for four-colour imaging: yESapphire, yECFP, yECitrine and tdimer2 exhibit little spectral overlap and can be easily distinguished, allowing labelling of multiple proteins within the same cell. These fluorescent proteins are

Table 5. Cross-talk between fluorophores in four-colour imaging (%)

		From			
		Sapphire	CFP	YFP	tdimer2
To	Sapphire		11	4.1	0
	CFP	1.5		0.3	0
	YFP	4.5	0		0
	tdimer2	0	0	0	

Strains with TDH3 tagged with the indicated fluorophores were mixed and imaged on a Zeiss Axiovert 200M. Cross-talk from the fluorophore-tagged strains into the other channels was calculated by linear regression of the pixel intensities from all cells tagged with a given fluorophore-tagged cell against the intensities measured for those pixels in the other fluorescence channels. The slope of this linear regression line gives the cross-talk between channels and is shown rounded to the nearest 0.1%.

also significantly brighter than those currently used for protein tagging in yeast, thereby improving the detection limit for tagged proteins. Our optimized YFP variants and GFP are about twice as bright as unoptimized fluorophores, and the DsRed variant tdimer2 is up to 15-fold brighter than unoptimized YFP. We recommend that the tdimer2 or yECitrine variants be used in future tagging projects to ensure maximum detectability of tagged proteins.

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