Brighter reporter genes from multimerized fluorescent proteins

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The discovery and development of the green fluorescent protein (GFP) has opened up new areas of biological research, particularly for the visualization of protein dynamics and gene expression in living cells and organisms (1,2). The development of wavelength-shifted variants of GFP has further expanded the impact on biological research. One of the fluorescent proteins’ most important applications has been as a tag for protein localization and turnover. Although attempts have been made to use XFPs [cyan fluorescent protein (CFP), GFP, and yellow fluorescent protein (YFP)] as reporters of promoter activity, these efforts have been less successful primarily because most promoters are not active enough to yield sufficient fluorescent protein accumulation to visualize without antibody enhancement (3–5). Nevertheless, almost all applications can be enhanced by improving the spectral yield of fluorescent proteins, which therefore has become an important research goal. For example, enhanced GFP (EGFP) (6) and Venus (7) have become widely used following their introduction, such that EGFP is currently the standard fluorescent protein used in biological applications.

Despite these developments, detection problems still remain an issue, especially when XFPs are used to report promoter activity in vivo in tissues from large multicellular organisms such as the mammalian central nervous system (CNS) (4,5). This is true whether fluorescent proteins are used as reporter genes directly following the promoter as well as when they are expressed as bicistronic messages following an internal ribosome entry site (IRES) sequence (8). In the latter case, gene expression downstream of the IRES is diminished, and fluorescence output is decreased or even absent in these constructs compared with that found in fusion proteins (9,10). Thus, improved fluorescent protein spectral yield will open up new possibilities for reporting spatial and temporal analysis of vertebrate gene expression in vivo.

As an attempt to improve the spectral yield of XFP fusion proteins, some investigations have employed tagging proteins of interest using multiple XFPs linked in tandem (11–13). However, despite the qualitative success of this approach for particular fusion proteins, no quantitative analysis of the increase in fluorescence output or the limits of this approach after multimerization has been performed. This work sought to quantitatively determine the improvements in spectral yield after XFP multimerization, with a particular emphasis on using these constructs as reporters of gene expression. Here we report quantitative and significant increases in fluorescence output after in-frame multimerization of XFP monomers. This enhancement is observed using a range of multimerized fluorescent proteins, effectively boosts spectral yield in IRES expression constructs, and can be employed using mammalian

Figure 1. Relative pixel intensity after EGFP multimerization. A549 cells were transfected with plasmids pEGFP-C1, p3xEGFP, or p6xEGFP (Table 1). (A) Pixel intensity in relative units after image analysis when all cells or the two brightest were considered. The pixel intensity recorded from cells transfected with pEGFP-C1 was set to 1 (panel A, white bars, labeled EGFP). Cells transfected with p3xEGFP yielded a 2.7-fold increase in luminosity over the single EGFP construct (panel A, light-gray bars, labeled 3xEGFP). Cells transfected with p6xEGFP displayed a slight increase in luminosity over single-copy EGFP (panel A, dark-gray bars, labeled 6xEGFP). In all cases, the values are expressed as the mean of the relative pixel intensity ±SEM. *, P < 0.001. (B–D) Micrograph of A549 cells transfected with pEGFP-C1, p3xEGFP, or p6xEGFP, respectively. Scale bar: 1 mm; insets: 20 μm. EGFP, enhanced green fluorescent protein.
promoters. Furthermore, using the enhanced yellow fluorescent protein (EYFP) variant Venus (7), we have generated the brightest fluorescent protein to date, underscoring that this simple approach will be generally applicable to all current and future monomeric fluorescent proteins.

We first constructed plasmids p3xEGFP and p6xEGFP, which express 3 or 6 copies in tandem of EGFP, respectively, under the cytomegalovirus (CMV) promoter (Table 1) (12). The plasmid pEGFP-C1 (BD Biosciences Clontech, Palo Alto, CA, USA) containing a single, CMV-driven EGFP copy, was used as a control (referred to as 1xEGFP). A549 cells (human lung adenocarcinoma; ATCC, Manassas, VA, USA) were transfected with these constructs using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA). Forty-eight-hour posttransfection cells were washed, fixed for 15 min in 4% paraformaldehyde in phosphate buffer, and mounted. For analysis of fluorescence intensity, photomicrographs were taken using the same digital settings for all transfection conditions (Figure 1, B–D), and cell luminosity was then quantitated using Adobe Photoshop®. The pixel intensity per unit area of individual cells was then quantitated using Adobe Photoshop® luminosity was then quantitated using Adobe Photoshop®. The pixel intensity per unit area of individual cells was then quantitated using Adobe Photoshop® luminosity was then quantitated using Adobe Photoshop®. The pixel intensity per unit area of individual cells was then quantitated using Adobe Photoshop® luminosity was then quantitated using Adobe Photoshop®. The pixel intensity per unit area of individual cells was then quantitated using Adobe Photoshop®

| Table 1. Multimerized Fluorescent Protein Plasmids Used for Transfection |
|-----------------------------|---------------------|-----------------|-----------------|
| Plasmid                     | Promoter            | Fluorescent    | No. of  |
|                             |                     | Protein-Encoded | Repeats | Comments |
| pEGFP-C1 (1xEGFP)ª          | CMVie               | EGFP           | 1       |          |
| p3xEGFP                     | CMVie               | EGFP           | 3       |          |
| p6xEGFP                     | CMVie               | EGFP           | 6       |          |
| pEGFP-ires-EYFP             | CMVie               | EYFP           | 1       | Bicistronic |
| pECFP-ires-3xEYFP           | CMVie               | EYFP           | 3       | Bicistronic |
| pfos-fos-EYFP               | mouse fos           | EYFP           | 1       | Fos fusion protein |
| pfos-fos-3xEYFP             | mouse fos           | EYFP           | 3       | Fos fusion protein |
| pEYFP                       | CMVie               | EYFP           | 1       |          |
| pVenusª                     | CMVie               | Venus          | 1       |          |
| p3xVenus                    | CMVie               | Venus          | 3       |          |

All plasmids except for pEGFP-C1 and pVenus were specifically constructed for this study. EGFP, enhanced green fluorescent protein; ECFP, enhanced cyan fluorescent protein; IRES, internal ribosome entry site; EYFP, enhanced yellow fluorescent protein. 

ªCommercially available (BD Biosciences Clontech, Palo Alto, CA, USA). 

ªEquivalent to Venus-PCS2 (kind gift from Dr. Atsushi Miyawaki, RIKEN Institute, Wako, Saitama, Japan).
by immunoblotting. Results showed that cells transfected with p6xEGFP displayed a series of lower molecular weight immunoreactive bands that may correspond to degradation fragments, consistent with the apparent decrease in fluorescence from hexameric EGFP. However, it should be noted that there may be some cell-type specificity to this limit (B.S. Glick, unpublished observations).

Next, we wanted to test whether enhanced fluorescence output from XFP multimerization could overcome detection limitations from typical reporter gene expression constructs using IRES-driven expression. Because the use of XFPs as protein tags can compromise protein function, reporter constructs carrying an IRES have frequently been used to report promoter activation without compromising the function of the endogenous protein (8). IRES sequences are able to drive translation in cap-independent mechanism (15) and, in general, the fluorescent protein is placed downstream of the IRES. However, the amount of IRES-driven reporter expressed is decreased relative to translation of the upstream gene, ranging from 20% to 50% of the first coding region (9), and in some cases reporter gene expression following the IRES sequence is undetectable (10). To evaluate the hypothesis that multimerization of XFPs improves detection of promoter activation in IRES expression systems, plasmids pECFP-ires-EYFP and pECFP-ires-3xEYFP (Table 1), with single and triple IRES-driven EYFP expression, respectively, were constructed. A549 cells were transfected, and fluorescence output was calculated as described above. Mean luminosity values from pECFP-ires-3xEYFP-transfected cells displayed a 2.5-fold increase compared with pECFP-ires-EYFP (Figure 2A, *, P < 0.001, Student’s unpaired t-test). These results show that this technique can be generalized to report gene expression in different experimental settings, and that consistent with our previous findings, fluorescence output approximately scales to the number of fluorescent protein monomers added.

We then tested whether XFP multimerization increases the ability to detect activation of an exemplar mammalian promoter, the c-fos promoter. Using the mouse c-fos promoter to drive expression of a Fos fusion protein (16), we compared spectral yield with single EYFP copy or with 3 tandem repeats of EYFP (Table 1, pfos-fosEYFP or pfos-fos3xEYFP). A549 cells were transfected; the mean luminosity values from cells receiving pfos-fos3xEYFP displayed a 2.4-fold increase when compared with cells transfected with pfos-fosEYFP (Figure 2B, *, P < 0.001, Student’s unpaired t-test). The increase in fluorescence obtained with a single promoter activation event was consistent with results obtained using XFP multimers driven by the constitutively active CMV promoter. In our studies, we did not observe any extranuclear fluorescence or any deleterious effects due to the increase in size of the reporter tag, and in general we have found that if a protein can tolerate a single GFP tag, it can usually tolerate a multimeric tag (17). However, we acknowledge that tripling the size of the tag might pose a problem for some applications. For this reason, we expect

![Figure 2](image-url)

**Figure 2.** Pixel intensity in different applications after fluorescent protein multimerization. (A) Fluorescent protein multimerization increased pixel intensity when expressed under the control of an IRES. A549 cells transfected with pECFP-ires-3xEYFP showed a 2.5-fold increase in pixel intensity as compared with cells transfected with single-copy pECFP-ires-1xEYFP. (B) Fluorescent protein multimerization increased pixel intensity when expressed under a typical mammalian promoter as a fusion protein. A549 cells transfected with pfos-fos3xEYFP displayed a 2.4-fold increase in pixel intensity as compared with cells transfected with pfos-fosEYFP. (C) Fluorescent protein multimerization increased pixel intensity when applied to “Venus,” a recently developed EYFP derivative. Cells transfected with (F) p3xVenus displayed a 12-fold increase in pixel intensity when compared with (D) cells transfected with the parental single-copy pEYFP. The increase in pixel intensity reached 2.7-fold when cells transfected with p3xVenus were compared with (E) the single-copy pVenus. Data show the mean of pixel intensity ± SEM. *, P < 0.001. Scale bars: 20 μm. IRES, internal ribosome entry site; EYFP, enhanced yellow fluorescent protein.
that the utility of this technology will be realized primarily as a reporter of promoter activity, especially in tissues from multicellular organisms.

One advantage of using in-frame fusion of multiple XFPs to enhance spectral output is that it can be general-
ized to future improvements in fluorescent protein output that increase brightness. To demonstrate this, we
multimerized Venus (7), an EYFP variant that exhibits improved spectral output and faster folding kinetics
compared with the parent fluorescent protein and is an important new tool for the expansion of fluorescent proteins in cell biology applications. A549 cells were transfected with pEYFP (Figure 2D), pVenus (Figure 2E), and p3xVenus (Figure 2F) (Table 1) as described above. Compared with a single copy of EYFP, we observed a more than 12-fold enhancement of fluorescence output using the 3xVenus construct (Figure 2C, *, P < 0.001, Student’s unpaired t-test). Consistent with previous results, p3xVenus was approximately 2.7-fold brighter than pVenus (Figure 2C). These results underscore the reliability and simplicity of our technology irrespective of the fluorescent protein or the experimental setting employed.

Finally, we examined photobleaching kinetics of the multimerized proteins. Cells transfected with pCMV-EYFP, pVenus, or p3xVenus were fixed and examined under continuous fluorescence excitation, imaging the same field once every minute. Fluorescence output was calculated, and data were plotted to determine the decay constant (tau) value for each construct. Although p3xVenus bleaches at a faster rate than p1xVenus or EYFP (tau = 5.7, 3.6, and 2.0 ms, respectively), even after 5 min of constant illumination, 3xVenus was still 8-fold brighter than pCMV-EYFP. These data further underscore the utility of multimerized XFPs under different experimental settings.

Fluorescent proteins have become an invaluable tool for imaging real-time events in living cells. The greatest success with XFPs has been achieved by using them as fluorescent tags for other proteins, particularly for cell biological applications. However, because fluorescent proteins do not amplify signal like enzymatic reporters such as β-galactosidase, their application as reporters of gene expression has been less useful. This is particularly true when fluorescent proteins have been employed as reporters of gene expression in the mouse CNS (4,5).

Here we report a technology that is widely applicable to virtually any current or future monomeric form of fluorescent protein when increased fluorescent output to report gene expression is required. In-frame fusion of multiple fluorescent protein monomers has previously been employed as protein tags in a limited number of applications (12,13); however, our report is the first quantitative, systematic investigation of fluorescent protein multimerization as a technique to enhance reporter gene fluorescent output. We have shown that multimerization scales with fluorescence output up to 3 tandem copies, that it can improve detection of single-promoter activation and IRES-coupled gene expression, and that it can be adapted to future improvements in fluorescence yield of XFPs. This application has produced the brightest fluorescent protein generated to date and constitutes an important advance in fluorescent protein development.

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COMPETING INTERESTS

STATEMENT

The authors declare no competing interests.

REFERENCES


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