Hexameric GFP and mCherry Reporters for the *Drosophila* GAL4, Q, and LexA Transcription Systems

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ABSTRACT The ability to distinguish cells and tissues of interest is critical for understanding their biological importance. In genetic model organisms, a prominent approach for discerning particular cells or tissues from others is the use of cell or tissue-specific enhancers to drive fluorescent reporters. This approach, however, is often limited by the brightness of the fluorescent reporter. To augment the ability to visualize cells or tissues of interest in *Drosophila melanogaster*, homo-hexameric GFP and mCherry reporters were developed for the GAL4, Q, and LexA transcription systems and functionally validated *in vivo*. The GFP and mCherry homo-hexameric fusion proteins exhibited significantly enhanced fluorescence as compared to monomeric fluorescent reporters and could be visualized by direct fluorescence throughout the cytoplasm of neurons, including the fine processes of axons and dendrites. These high-sensitivity fluorescent reporters of cell morphology can be utilized for a variety of purposes, especially facilitating fluorescence-based genetic screens for cell morphology phenotypes. These results suggest that the strategy of fusing monomeric fluorescent proteins in tandem to enhance brightness should be generalizable to other fluorescent proteins and other genetic model organisms.

SINCE the cloning of GFP >20 years ago (Prasher *et al.* 1992) fluorescent proteins have become ubiquitous in biomedical research. Fluorescent proteins are used for a wide variety of purposes including as markers of gene expression (Chalfie *et al.* 1994), protein localization dynamics (Edwards *et al.* 1997), markers of cell morphology (Lee and Luo 1999), markers of subcellular organelles (Rizzuto *et al.* 1995; Chi *et al.* 2001), indicators of cellular activity (Miyawaki *et al.* 1997; Chen *et al.* 2013), and even reporters of neuronal connectivity (Feinberg *et al.* 2008). Although their immense value as tools in biological research is beyond question, a limiting factor in their usefulness is frequently how bright they are. The reason brighter fluorescent proteins are desirable is simple: they are easier to detect and therefore yield more sensitive assays. Consequently, since the earliest days of the use of fluorescent proteins in genetically manipulable organ-

isms, there has been a continuing effort to identify brighter fluorescent proteins via mutagenesis (Heim *et al.* 1995; Shaner *et al.* 2004; Goedhart *et al.* 2012).

In addition to enhancing the intrinsic brightness of fluorescent proteins through mutagenesis strategies, other approaches for producing brighter fluorescent reporters include using multiple copies of fluorescent transgenes (Halfhill *et al.* 2003), bicistronic transcripts (Halfon *et al.* 2002), transcription and translation enhancement (Pfeiffer *et al.* 2010, 2012), and mining for novel fluorescent proteins in the natural world (Hunt *et al.* 2010; Shaner *et al.* 2013). To varying degrees, all of these strategies have proven successful. However, given the extensive historical efforts to augment the observed brightness of fluorescent proteins either intrinsically or otherwise, further utilization of these strategies to identify significantly brighter fluorescent proteins may well have reached a point of diminishing returns.

We have thus taken an alternative “tandem fusion” approach to enhance the brightness of fluorescent proteins that involves fusing monomers of fluorescent proteins to each other in tandem into single hexameric proteins. To assess the effectiveness of the approach, we expressed the tandem homo-hexamers of GFP and mCherry in *Drosophila*. Quantitative comparison between hexameric GFP and monomeric GFP suggests that the brightness of tandem fluorescent

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protein multimers is directly correlated with the number of repeats. Homo-hexamers of GFP and mCherry distribute throughout the cytoplasm and are thus useful as markers of cell morphology. This work demonstrates the validity of the tandem fusion approach as a complement to traditional strategies for enhancing the brightness of fluorescent proteins.

Materials and Methods

Entry and expression clone construction

The L5-syn21eGFP-L4 entry clone was generated by pairing the attB5syn21eGFP oligo 5'-GGGGACAACCTTTGTATACAA AAGTTGGCAACTTAAAAAATCAAAATGGTGAGCAAGG GCGAGGAGCTGTTC-3' with the attB4eGFP oligo 5'-GGGGACAACCTTTGTATAGAAAAGTTGGGTGCTTGTACAGC TCGTCCATGCCGAGAGTGAT-3' in a PCR reaction using enhanced GFP (eGFP) as a template and combining the resulting ~800-bp band in a BP reaction with the donor vector pDONR 221 P5-P4. The L5-syn21mCherry-L4 entry clone was generated similarly except in the PCR reaction an mCherry template was used in combination with the attB5syn21mCherry oligo 5'-GGGGACAACCTTTGTATACAAAAGTTGGCAACTTAAAAAATCAAAATGTTGAGCAAGGGCGAGGAGGATAAC-3' and the attB4mCherry oligo 5'-GGGGACAACCTTTGTATAGAAAAGTTG GGTGCTTGTACAGCTCGTCCATGCCGC-3'. Details of the BP and LR reactions have been previously described (Petersen and Stowers 2011). Both entry clones were sequenced in their entirety and determined to be free of PCR-generated mutations. The complete sequences of both entry clones are available upon request. All entry clones used in the LR reactions, except for the L5-syn21eGFP-L4 and L5-syn21mCherry-L4 entry clones described above, have been previously described (Petersen and Stowers 2011; Shearin *et al.* 2013). The L3-4XeGFP-L3 entry clone used here is the same as the entry clone previously described as L3-4XeGFP-MYC-L3. Reanalysis of the Myc-tag region of this entry clone revealed a previously unrecognized deletion of a leucine codon within the Myc-tag sequence. Since the Myc-tag sequence is aberrant and is not recognized with anti-Myc antibodies using immunohistochemistry (Petersen and Stowers 2011) or Western blotting (data not shown), the Myc label has been omitted.

Fly stocks

The 20XUAS-6XGFP, 10XQUAS-6XGFP, 13XLexAop2-6XGFP, 20XUAS-6XmCherry, 10XQUAS-6XmCherry, and 13XLexAop2-6XmCherry fly stocks were obtained by *PhiC31*-mediated transformation of their corresponding expression constructs into the VK00018 (Venken *et al.* 2006) and attP2 (Groth *et al.* 2004) landing sites by Bestgene, Inc. (Table 1). References for the other fly strains used in this study are as follows: *nompC-GAL4* and *nompC-QF* (Petersen and Stowers 2011), *nompC-LexA* and *n-syb-LexA* (Shearin *et al.* 2013), 20XUAS-GFP (Pfeiffer *et al.* 2012), *UAS-mCD8GFP* (Lee and Luo 1999), *n-syb-GAL4* (Bushey *et al.* 2009), 24B-GAL4 (Brand and Perrimon 1993), 5XQUAS-mCD8GFP and

Table 1 Fly strains and landing-site locations

Strains	Landing sites
20XUAS-6XGFP	VK00018, attP2
10XQUAS-6XGFP	VK00018, attP2
13XLexAop2-6XGFP	VK00018, attP2
20XUAS-6XmCherry-HA	VK00018, attP2
10XQUAS-6XmCherry-HA	VK00018, attP2
13XLexAop2-6XmCherry-HA	VK00018, attP2

5XQUAS-mtdTom-3XHA (Potter *et al.* 2010), 10XUAS-IVS-mCD8RFP and 13XLexAop2-mCD8GFP (Pfeiffer *et al.* 2010), α -tubulin-QF2w and *n-syb-QF2* (gifts from Chris Potter, The Johns Hopkins University School of Medicine).

Fluorescence imaging

All images were captured within 30 min of dissection on a Leica SP5 confocal microscope via direct fluorescence with 488 nm (GFP) or 561 nm [red fluorescent proteins (RFP) mCherry, RFP, and tdTomato] laser excitation.

Image quantitation

To quantitate the difference in fluorescence between genotypes, 3D surface reconstructions of 10× z-stacks captured using uniform confocal settings were rendered using identical parameters in Imarus image analysis software. The total volume of fluorescing voxels was calculated for each z-stack and averaged across five animals per genotype. Examples of rendered images used for quantitation in Figure 5 are shown in Supporting Information, Figure S4.

Western blots

Adult *Drosophila* were processed for Western blotting as previously described (Koelle *et al.* 1991). Signals were detected via chemiluminescence and generated using Immuno-Star HRP (BioRad). Primary antibodies were rabbit anti-GFP Abfinity mAb (Invitrogen catalog #G10362; 1:500), rat anti-HA mAb 3F10 (Roche-Cat # 11 867 423 001; 1:500), and mouse anti-Dlg (1:50) (Parnas *et al.* 2001). Secondary antibodies were goat anti-rabbit-HRP (Jackson catalog #111-035-144; 1:2500), donkey anti-rat-HRP (Jackson catalog #712-035-153; 1:2500), and goat anti-mouse-HRP (Jackson catalog #115-035-146; 1:2500).

Results

Construction of tandem 6XGFP and 6XmCherry transgenes

Drosophila expression constructs containing homo-hexameric tandem fusions of eGFP (hereafter GFP) and mCherry were assembled using Gateway MultiSite cloning (Cheo *et al.* 2004; Petersen and Stowers 2011). A recent report characterizing different 5' and 3' untranslated (UTR) sequences determined that, in combination, the Syn21 5' UTR and *p10* 3' UTR increased the expression of a cytoplasmic GFP reporter up to 20-fold as compared to traditional 5' and

3' UTRs (Pfeiffer *et al.* 2012). In an attempt to produce even brighter fluorescent reporters, the translation enhancement strategy of including the Syn21 5' UTR and *p10* 3' UTR was complemented by a tandem fusion strategy in which a total of six *Drosophila* transgenes were generated containing tandem homo-hexameric fusions of GFP or mCherry under control of UAS, QUAS, or LexAop2 operator sequences. A schematic diagram for the assembly of the 20XUAS-6XGFP reporter is shown in Figure 1A. Details of the Gateway MultiSite LR reactions used to assemble 20XUAS-6XGFP (Figure 1B), 10XQUAS-6XGFP (Figure 1C), 13XLexAop2-6XGFP (Figure 1D), 20XUAS-6XmCherry-HA (Figure 1E), 10XQUAS-6XmCherry-HA (Figure 1F), and 13XLexAop2-6XmCherry-HA (Figure 1G) are described in the legend of Figure 1. Restriction digests of the assembled expression clones yielded fragments of the predicted sizes (Figure S1).

Full-length hexameric GFP and mCherry-HA proteins are expressed

To determine if the expected full-length hexameric GFP and mCherry-HA proteins were being expressed, Western analysis was performed. Each of the six transgenes shown schematically in Figures 1, B–G were first inserted into both the *VK00018* and the *attP2* landing sites on the second and third chromosomes (Table 1), respectively, and subsequently expressed using transcription system appropriate *n-syb* drivers. Protein extracts from these flies were then subjected to Western analysis. Anti-GFP antibodies detected a prominent band between 140 and 240 kDa for all three hexameric GFP expression constructs at both landing sites (Figure S2A), consistent with the expected molecular weight of ~170 kDa for hexameric GFP.

Similarly, an anti-HA antibody detected a prominent band between 140 and 240 kDa, consistent with the expected molecular weight of ~170 kDa for hexameric mCherry for all three hexameric mCherry expression constructs at both landing sites (Figure S2B). Since the HA tag is present only on the carboxy-terminal mCherry and anti-HA antibodies recognize the HA epitope on the hexameric mCherry expression constructs, this is additional confirmation that full-length hexameric mCherry is being expressed. The Western analysis results thus indicate that the expected full-length hexameric GFP and mCherry-HA proteins are being produced from all expression constructs for all three transcription systems.

6XGFP and 6XmCherry-HA proteins are functional reporters of neuronal morphology

To characterize the fluorescence and cellular distribution properties of the 6XGFP and 6XmCherry-HA expression constructs, they were expressed using transcription-system-appropriate *nompC* drivers. The *nompC* drivers for all three *Drosophila* binary transcription systems have been previously shown to exhibit expression in the chordotonal organs and class III sensory neurons of third instar larva (Petersen and Stowers 2011; Shearin *et al.* 2013). Class III sensory neurons are bipolar neurons with easily distinguished axons and dendrites. Their low density in third instar larva makes individual

neurons readily discernible, thus making them especially suitable for assessing the intraneuronal distribution properties of the 6XGFP and 6XmCherry-HA proteins.

All second and third chromosome inserts of the 6XGFP and 6XmCherry-HA constructs (Figure 2, A–F) exhibited expression in third instar larval class III sensory neurons and chordotonal organs using *nompC* drivers. These results demonstrate the 6XGFP and 6XmCherry-HA reporters for all three transcription systems at both landing sites are functionally fluorescent. GFP and mCherry fluorescence was observed throughout the class III sensory neurons including axons (large arrowheads), dendrites (small arrowheads), cell bodies (small arrows), and presynaptic terminals (large arrows) (arrows and arrowheads shown only in Figure 2A and Figure 3A). The GFP and mCherry fluorescence intensities correlate with approximate cytoplasmic volumes for each neuronal region, suggesting that the 6XGFP and 6XmCherry-HA proteins localize to the cytoplasm, as expected based on the cytoplasmic localization of monomeric GFP (Pines 1995). The distribution of hexameric GFP within axons and dendrites is more readily observed in higher-magnification images (Figure S3, A and B) and does not appear to be different from monomeric GFP (Figure S3, C and D). The broad distribution of hexameric GFP and hexameric mCherry-HA throughout all cytoplasmic compartments of neurons, including the fine processes of axons and dendrites, establishes their utility as markers of neuronal morphology.

Hexameric GFP and mCherry reporters exhibit enhanced brightness as compared to existing alternatives

How does the relative brightness of the hexameric GFP and mCherry reporters compare to existing alternatives? For each of the six hexameric GFP and mCherry reporters except 13XLexAop2-6XmCherry-HA (for which no analogous alternative was available), an existing cytoplasmic or plasma membrane-associated GFP or red fluorescent reporter was chosen for quantitative pair-wise comparison. For each pair-wise comparison, identical confocal settings were used for image acquisition and the same transcription system-appropriate *nompC* driver was used for expression to eliminate the driver as a source of variability. Confocal settings, however, were not identical between different pair-wise comparisons, and thus it is not valid to make inferences as to quantitative differences in brightness between pairs from these data. To quantitate the differences in fluorescent signal between different genotypes, Imaris software was used to render 3D surface reconstructions from which total volumes were calculated. Summation of volume from 3D renderings was chosen as a quantitation method because it directly correlates with the fluorescence signal from observable structures and is therefore a biologically relevant measure of the utility of a reporter.

The 20XUAS-6XGFP reporter (Figure 4, A and D) was compared to both the monomeric cytoplasmic GFP reporter 20XUAS-GFP (Figure 4B) and the widely used plasma membrane reporter UAS-mCD8GFP (Figure 4E). Quantitation of

Figure 1 Assembly of the 6XGFP and 6XmCherry expression constructs. (A) Four-fragment Gateway MultiSite recombination cloning was used to assemble the 20XUAS-6XGFP expression construct. The L1-20XUAS-R5, L5-Syn21-GFP-L4, R4-GFP-R3, and L3-4XGFP-L2 entry clones were combined with the pDESTp10aw destination vector in an LR reaction in which five separate site-specific recombination reactions resulted in the insertion of a 20XUAS operator sequence upstream of six tandem in-frame fusions of GFP into the pDESTp10aw destination vector. (B) The 20XUAS-GFP expression construct resulting from the LR reaction shown in A. (C) The 10XQUAS-6XGFP and (D) 13XLexAop2-6XGFP expression constructs resulting from substitution of L1-10XQUAS-R5 and 13XLexAop2 entry clones, respectively, for the L1-20XUAS-R5 entry clone in the LR reaction shown in A. The (E) 20XUAS-6XmCherry-HA, (F) 10XQUAS-6XmCherry-HA, and (G) 13XLexAop2-6XmCherry-HA expression constructs resulting from substitution of analogous mCherry entry clones for the GFP entry clones in the LR reactions used to produce B, C, and D, respectively. All expression constructs contain a Syn21 5' UTR immediately upstream of the start codon of the amino-terminal GFP or mCherry and a p10 3' UTR downstream of the attL2/attR2 recombination site to enhance the efficiency of the recombination and thereby maximize expression levels. The pDESTp10aw destination vector contains a mini-white transgenesis marker and a *PhiC31 attB* recombination site for targeted transgenesis as indicated in A.

fluorescent signals yielded means (in $10^6 \mu\text{m}^3$) of 3.24 ± 0.11 SEM for 20XUAS-6XGFP, 0.596 ± 0.046 SEM for 20XUAS-GFP, and 0.224 ± 0.023 SEM for UAS-mCD8GFP (Figure 4, C and F). Total fluorescent signals for 20XUAS-6XGFP were thus $5.43\times$ and $14.4\times$ greater than 20XUAS-GFP and UAS-mCD8GFP, respectively. Qualitatively similar differences in fluorescence intensity were also observed in higher magnification images of cell bodies, axons, and dendrites (Figure S3; A, B-20XUAS-6XGFP; C, D-20XUAS-GFP; E, F-UAS-mCD8GFP).

Similarly, quantitative pair-wise comparisons were also made between the 10XQUAS-6XGFP (Figure 4G), 13XLexAop2-6XGFP (Figure 4J), 20XUAS-6XmCherry (Figure 5A), and 10XQUAS-6XmCherry (Figure 5D) reporters and the existing plasma membrane reporters 5XQUAS-mCD8GFP (Figure 4H), 13XLexAop2-mCD8GFP (Figure 4K), 10XUAS-mCD8RFP (Figure 5B), and 5XQUAS-mtdTomato-3XHA (Figure 5E), respectively. Quantitative fluorescent signals were determined (in $10^6 \mu\text{m}^3$) as 1.76 ± 0.11 SEM for 10XQUAS-6XGFP and

Figure 2 Functional demonstration of the 6XGFP expression constructs. Direct GFP fluorescence imaging of third instar larva-containing transcription-system-compatible *nompC* drivers and the indicated 6XGFP reporters. In third instar larva, the *nompC* drivers express specifically in chordotonal organs and class III sensory neurons. (A) *yw*; 20XUAS-6XGFP/+; *nompC-GAL4*/+. (B) *yw*; *nompC-GAL4*/+; 20XUAS-6XGFP/+. (C) *yw*; 10XQUAS-6XGFP/+; *nompC-QF*/+. (D) *yw*; *nompC-QF*/+; 10XQUAS-6XGFP/+. (E) *yw*; 13LexAop2-6XGFP/+; *nompC-LexA*/+. (F) *yw*; *nompC-LexA*/+; 13LexAop2-6XGFP/+. All images were acquired using identical confocal settings. In A, the small arrowhead indicates a dendrite, the small arrow a cell body, the large arrowhead an axon bundle, and the large arrow presynaptic terminals of chordotonal organs and class III sensory neurons. These data demonstrate that both the second (*VK00018*) and third chromosome (*attP2*) insertions of all three 6XGFP expression constructs are functional and that hexameric GFP distributes throughout the cytoplasm of neurons. II, *VK00018* landing site; III, *attP2* landing site. Images were collected with a 10× water immersion objective. Bar, 500, μ m.

0.0344 ± 0.0018 SEM for 5XQUAS-mCD8GFP (Figure 4I), a 51.3× difference; 2.01 ± 0.039 SEM for 13LexAop2-6XGFP and 0.0830 ± 0.0083 SEM for 13LexAop2-mCD8GFP (Figure 4L), a 24.2× difference; 2.72 ± 0.052 SEM for 20XUAS-6xmCherry and 0.0180 ± 0.0058 SEM for 10XQUAS-mCD8RFP (Figure 5C), a 151× difference; and 4.24 ± 0.29 SEM for 10XQUAS-6XmCherry and 1.29 ± 0.29 SEM for 5XQUAS-mtdTomato-3XHA (Figure 5F), a 3.28× difference. Major differences in expression between the hexameric mCherry

Figure 3 Functional demonstration of the 6XmCherry-HA expression constructs. Direct mCherry fluorescence imaging of third instar larva-containing transcription-system-compatible *nompC* drivers and the indicated 6XmCherry reporters. In third instar larva, the *nompC* drivers express specifically in chordotonal organs and class III sensory neurons. (A) *yw*; 20XUAS-6XmCherry-HA/+; *nompC-GAL4*/+. (B) *yw*; *nompC-GAL4*/+; 20XUAS-6XmCherry-HA/+. (C) *yw*; 10XQUAS-6XmCherry-HA/+; *nompC-QF*/+. (D) *yw*; *nompC-QF*/+; 10XQUAS-6XmCherry-HA/+. (E) *yw*; 13LexAop2-6XmCherry-HA/+; *nompC-LexA*/+. (F) *yw*; *nompC-LexA*/+; 13LexAop2-6XmCherry-HA/+. All images were acquired using identical confocal settings. In A, the small arrowhead indicates a dendrite, the small arrow a cell body, the large arrowhead an axon bundle, and the large arrow presynaptic terminals of chordotonal organs and class III sensory neurons. These data demonstrate that both the second (*VK00018*) and the third chromosome (*attP2*) insertions of all three 6XmCherry-HA expression constructs are functional and that hexameric mCherry-HA distributes throughout the cytoplasm of neurons. II, *VK00018* landing site; III, *attP2* landing site. Images were collected with a 10× water immersion objective. Bar, 500 μ m.

reporters and alternatives are also apparent with pan-neuronal drivers (Figure S5).

In addition to the difference in the number of GFP or RFP monomers, a number of factors likely contribute to the observed quantitative differences in fluorescent signal in these pair-wise comparisons. These factors include different genomic locations (in some, but not all, cases), different 5' and 3' UTRs, different numbers of repeats of transcription system operator elements

Figure 4 Quantitative pair-wise comparison of fluorescence signals between the hexameric GFP reporters and existing alternatives. (A) *yw; nompC-GAL4/+; 20XUAS-6XGFP/+*. (B) *yw; nompC-GAL4/+; 20XUAS-GFP/+*. (C) Bar graph of fluorescent signals comparing A and B. (D) *yw; nompC-GAL4/+; 20XUAS-GFP/+*. (E) *yw; nompC-GAL4/+; UAS-mCD8GFP/+*. (F) Bar graph of fluorescent signals comparing D and E. (G) *yw; nompC-QF/+; 10XQUAS-6XGFP/+*. (H) *yw; nompC-QF/+; 5XQUAS-mCD8GFP/+*. (I) Bar graph of fluorescent signals comparing G and H. (J) *yw; nompC-LexA/+; 13XLexAop2-6XGFP/+*. (K) *yw; nompC-LexA/+; 13XLexAop2-mCD8GFP/+*. (L) Bar graph of fluorescent signals comparing J and K. All images were acquired as direct fluorescence from wandering third instar larva with a 10× water immersion objective using identical confocal settings within a given pair-wise comparison. Total fluorescent signals from each reporter were determined from 3D surface reconstructions generated using Imaris software. Fold differences are shown in the upper right quadrant of C, F, I, and L. Numerical data on which the bar graphs are based is presented in the text. Error bars are SEM. Bar, 500 μm.

(e.g., 10XQUAS vs. 5XQUAS), and cytoplasmic vs. plasma membrane localization. The one exception is the pair-wise comparison of 20XUAS-6XGFP to 20XUAS-GFP where none of these factors is different between the two reporters. In this case, the observed 5.43× difference in fluorescent signal between hexameric GFP and monomeric GFP suggests a linear relationship between the number of fluorescent protein monomers and the magnitude of fluorescent signal. Regardless of the explanations for the observed differences in fluorescent signal in the pair-wise comparisons, this quantitative analysis demonstrates that, in all pair-wise comparisons examined, the hexameric GFP and mCherry reporters exhibit significantly enhanced brightness as compared to existing alternatives.

Hexameric GFP and mCherry reporters can be observed with the naked eye

The hexameric GFP and mCherry reporters exhibit such strong fluorescence that they can be directly visualized at low

magnification with a nonfluorescent stereomicroscope using standard white light illumination or, in some cases even with the naked eye, when driven by strong and/or broadly expressing drivers. As an example, when using the pan-neuronal driver *n-syb-GAL4*, the purple hue of the 20XUAS-6XmCherry-HA reporter can be distinguished in the larval CNS (arrows, Figure 6, C and D) from wild-type controls (arrows, Figure 6, A and B) both before and after dissection. As a second example, the purple color of the 20XUAS-6XmCherry-HA reporter is even more apparent when driven by the muscle-specific driver 24B-GAL4 before (Figure 6E) or after (Figure 6F) dissection. The purple hue of the 6XmCherry-HA reporter can also be distinguished in 24B-GAL4, UAS-6XmCherry-HA (Figure 6H) adults as compared to a wild-type control (Figure 6G). When expressed using a ubiquitous driver, the 6XGFP reporter can also be observed without a fluorescence microscope in larva (Figure S6B; compare to Figure S6A) and adults (Figure S6E; compare to

Figure 5 Quantitative pair-wise comparison of fluorescence signals between the hexameric mCherry-HA reporters and existing alternatives. (A) *yw; nompC-GAL4/+; 20XUAS-6XmCherry-HA/+*. (B) *yw; nompC-GAL4/+; 10XUAS-mCD8RFP/+*. (C) Bar graph of fluorescence signals comparing A and B. (D) *yw; nompC-QF/+; 10XQUAS-6XmCherry-HA/+*. (E) *yw; nompC-QF/+; 5XQUAS-mtdTomato-3XHA/+*. (F) Bar graph of fluorescence signals comparing D and E. All images were acquired as direct fluorescence from wandering third instar larva with a 10× water immersion objective using identical confocal settings. Total fluorescent signals from each reporter were determined from 3D surface reconstructions generated using Imaris software. Fold differences are shown in the upper right quadrant of C and F. Numerical data on which the bar graphs are based are presented in the text. Error bars are SEM. Bar, 500 μm.

Figure S6D). The ability to visualize the hexameric GFP and mCherry reporters with white light when expressed using strong or broadly expressing drivers obviates the need for a fluorescence microscope to select animals of the desired genotype in some instances.

Toxicity assessment of the hexameric GFP and mCherry proteins

To assess whether the hexameric GFP and mCherry reporters exhibit toxicity, they were ubiquitously expressed using the α -tubulin-QF2w driver. Larvae (Figure S6B) and adults (Figure S6E) doubly heterozygous for α -tubulin-QF2w and 10XQUAS-6XGFP exhibited little, if any, lethality. However, attempts to homozygote either the driver or the reporter resulted in early larval lethality. Animals doubly heterozygous for α -tubulin-QF2w and 10XQUAS-6XmCherry-HA exhibited little, if any, lethality through the third instar larval stage of development (Figure S6C), but the vast majority of doubly heterozygous animals of this genotype died during pupal development. A rare doubly heterozygous “escaper” adult is shown in Figure S6F. Although no lethality or other obvious phenotypes have been observed with any other driver besides α -tubulin-QF2w, these results indicate that the hexameric GFP and mCherry reporters do exhibit a low level of toxicity that upon high level, ubiquitous expression can elicit lethality.

Discussion

Tandem fusions enhance visibility in proportion to copy number

This investigation demonstrates the feasibility of fusing monomers of fluorescent proteins in tandem to enhance the brightness of fluorescent reporters. Quantitative analysis revealed a 5.43× enhancement in fluorescent signal with 20XUAS-6XGFP as compared to 20XUAS-GFP (Figure 5C). This finding suggests that most, if not all, of the GFP mono-

mers within the hexameric GFP retain their functional ability to fluoresce since the observed signal is nearly equivalent to the six-fold difference in GFP copy number between the two constructs. Fusing GFPs to each other in tandem thus does not seem to disrupt the capacity of the component monomers to fluoresce. The enhanced brightness of the 6XmCherry-HA reporters to existing alternatives also implies that most, if not all, of the constituent mCherry monomers retain their capacity for fluorescence. These results with hexameric GFP and hexameric mCherry suggest that the tandem fusion approach should be feasible for other fluorescent proteins.

Hexameric GFP and mCherry are not diffusion limited

Fundamental chemical and physical principles dictate that larger molecules diffuse more slowly than smaller ones. It was thus possible the distribution of hexameric GFP and hexameric mCherry would be diffusion limited because of their relatively large sizes and they therefore would predominantly localize in or near sites of translation in the cell body. However, the observed distribution of hexameric GFP and hexameric mCherry seems approximately proportional with the amount of cytoplasm in any given region of a neuron. The observation that hexameric GFP and hexameric mCherry proteins disperse throughout neurons, including the fine processes of axons and dendrites, thus indicates the sizes of hexameric GFP and hexameric mCherry do not limit their distribution within the cytoplasm.

Whether hexameric GFP and hexameric mCherry distribute throughout the cytoplasm by diffusion alone or whether an active transport process may also be involved is an open question. The observation that puncta of hexameric GFP (Figure S3, A and B) are observed in axons and dendrites implies that at least some hexameric GFP may be actively transported along filaments in axons and dendrites. Similar puncta are also observed for monomeric GFP (Figure S3, C

Figure 6 The 6XmCherry-HA reporter can be observed directly in larvae and adults without a fluorescence microscope. No purple color is observed in wild-type (+/+) control larva (A) before or (B) after dissection. The purple hue of the 6XmCherry-HA reporter can be distinguished in the larval CNS with a pan-neuronal driver (C) before and (D) after dissection in *yw; n-syb-GAL4/+; 20XUAS-6XmCherry-HA/20XUAS-6XmCherry-HA* larva. Arrows in A–D indicate the location of the larval CNS. The purple hue of the 6XmCherry-HA reporter is observed throughout the larva with a muscle driver (E) before and (F) after dissection in *yw; 24B-GAL4; 20XUAS-6XmCherry-HA/24B-GAL4*, and *20XUAS-6XmCherry-HA* animals. No purple color is apparent in (G) *Canton-S* wild-type (+/+) control adult animals, but the purple hue of the 6XmCherry reporter can be discerned in adult (H) *yw; 24B-GAL4; 20XUAS-6XmCherry-HA/24B-GAL4*, and *20XUAS-6XmCherry-HA* adults with a muscle driver. With drivers that express strongly or broadly enough, the genotype of animals expressing the 6XmCherry-HA reporter can be identified without a fluorescent microscope.

and D), suggesting that whatever the cause of the puncta formation, the neurons are not processing hexameric GFP differently from monomeric GFP. Regardless of the relative importance of diffusion and active transport in distributing hexameric GFP or hexameric mCherry within neurons, their nearly proportional distribution throughout the cytoplasm with minimal preferential accumulation at specific locations makes them suitable as markers of cell morphology.

Toxicity of the hexameric GFP and mCherry proteins

The hexameric GFP and mCherry proteins were determined to cause lethality when expressed ubiquitously. It is not clear

whether the lethality is due to one or more specific cell or tissue types being especially sensitive to hexameric GFP and mCherry or if there is some cumulative threshold level of expression summed across the entire organism above which lethality results. Flies heterozygous for the pan-neuronal driver *n-syb-GAL4* and homozygous for *20XUAS-6XmCherry* (Figure 6, C and D) are viable with no obvious abnormalities such as developmental delay (the *n-syb-GAL4* driver alone is not viable as a homozygote so it could not be determined if doubly homozygous animals are viable). Flies doubly homozygous for the pan-muscle driver *24B-GAL4* and *20XUAS-6XmCherry* (Figure 6, E, F, and H) are also viable with no obvious phenotypes. These results indicate that neurons and muscles can tolerate high-level expression of hexameric mCherry without apparent consequence. Thus, the hexameric mCherry protein is not a potent toxin for just any cell type. The lethality results nevertheless indicate that the hexameric GFP and mCherry proteins do exhibit some low level of toxicity that may make them incompatible with a small subset of drivers that exhibit broad, high-level expression or expression in hypersensitive cells or tissues.

Applications of the 6XGFP and 6XmCherry reporters

The 6XGFP and 6XmCherry reporters for the GAL4, LexA, and Q binary transcription systems described here will be of immediate use to *Drosophila* researchers as markers of cell morphology for applications where enhanced brightness is advantageous. The ease with which we were able to visualize the fine processes of axons and dendrites with the 6XGFP and 6XmCherry-HA reporters using direct fluorescence suggests that they should be useful markers for any cell type since few, if any, other cell types have morphological features with lower cytoplasm-to-plasma membrane ratios than those found in the axons and dendrites of neurons.

One major potential application of these fluorescent reporters is enhancing the feasibility of genetic screens. The increased brightness of these reporters facilitates the observation of cells and tissues of interest, thereby augmenting the sensitivity of assays aimed at detecting phenotypic abnormalities in the morphology of cells marked with these reporters. Practically, this enhanced feasibility could translate into being able to conduct a genetic screen on intact animals using a stereomicroscope that, with a dimmer fluorescent reporter, would require labor-intensive dissection and immunostaining before assaying on a confocal microscope. These reporters will be especially useful for conducting genetic screens on *Drosophila* larva with their nearly translucent cuticle, but they should also prove useful for genetic screens on adult *Drosophila* whose more opaque cuticle has previously been a limitation for fluorescence-based genetic screens.

Future prospects

While hexamers of GFP and mCherry were examined in this investigation, there is no theoretical reason that expression constructs encoding tandem fluorescent protein multimers with larger numbers of repeats could not be assembled that

would exhibit even brighter fluorescence. Rather than an intrinsic upper limit on the number of monomers that would still yield a functional and diffusible tandem fluorescent protein multimer, the limiting factor is more likely to be the practical ability to assemble such constructs. Traditional restriction enzyme cloning is not ideally suited for combining more than one or two DNA fragments at a time into an expression vector and is thus suboptimal for constructing fluorescent protein multimers containing large numbers of repeats. More recently developed cloning methods including Gateway MultiSite (Cheo *et al.* 2004) and Golden Gate (Engler *et al.* 2009), which has been used to assemble up to 31 DNA fragments into a vector for construction of TALENs (Cermak *et al.* 2011), are capable of combining larger numbers of DNA fragments in a single step and are thus more suited for assembling tandem fluorescent protein multimers. Using these, or other yet-to-be-developed cloning strategies, alone or in combination, will undoubtedly enable the assembly of expression constructs encoding even brighter tandem fluorescent proteins consisting of higher repeat multimers.

Our success in fusing monomers of fluorescent proteins in tandem suggests that tandem fusions might also be successful with other types of proteins, particularly cytoplasmic proteins, whose enhanced expression would be desirable. This includes nonfluorescent reporters such as horseradish peroxidase or alkaline phosphatase, genetically encoded calcium indicators, various types of cytoplasmic enzymes, and any number of other cytoplasmic proteins.

It should also be feasible to fuse fluorescent protein multimers to specific proteins of interest. The resulting bright, fluorescent fusion proteins would be useful for investigating protein localization dynamics and for facilitating genetic screens designed to elucidate the function and/or pathway of the protein of interest.

Finally, although we have demonstrated the utility of the tandem fusion approach to produce bright fluorescent markers of cell morphology in *Drosophila*, it is potentially applicable to any genetically manipulable model organism including worms, zebrafish, and mice. Similarly bright fluorescent reporters in other model systems could be useful for the same purposes as discussed above for *Drosophila*.

Acknowledgments

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GENETICS

Supporting Information

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Hexameric GFP and mCherry Reporters for the *Drosophila* GAL4, Q, and LexA Transcription Systems

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Figure S1 Ethidium bromide stained agarose gel of the 6XeGFP and 6XmCherry-HA expression constructs. Each of the indicated six expression constructs was double digested with *Xho* I and *Hind* III before electrophoresis on a 0.7% agarose gel. The 7.5kb vector band in each lane and the insert bands between 5.0 and 5.5kb were as predicted.

Figure S2 Western analysis indicates full-length hexameric GFP and mCherry-HA proteins are being expressed. Transcription system appropriate n-syb drivers that express pan-neuronally were combined with the indicated expression constructs to generate doubly heterozygous adult flies that served as the source of the protein extracts. (A) Western blot of all three hexameric GFP expression constructs at both landing sites probed with an anti-GFP antibody. A prominent band consistent with the predicted ~170kd molecular weight of hexameric GFP is observed. (B) Western blot of all three hexameric mCherry-HA expression constructs at both landing sites probed with an anti-HA antibody. A prominent band consistent with the predicted ~170kd molecular weight of hexameric mCherry-HA is observed. Lower molecular weight bands in both blots appear to be breakdown products of the full-length hexameric GFP and mCherry-HA proteins. The presence of prominent bands at the predicted molecular weight for hexameric GFP and mCherry-HA demonstrates full-length hexameric proteins are being produced by all three constructs at both landing sites. Duplicate Western blots of the same protein extracts were run on separate gels and probed with anti-Dlg as a loading control and are shown at the bottom of each blot. (II)-second chromosome insert. (III)-third chromosome insert.

Figure S3 The fine processes of axons and dendrites can be distinguished with the 6XGFP reporter. Direct fluorescence imaging of class III sensory neurons and chordotonal organs in third instar larva at high magnification. (A, B) *yw; nompC-*

*GAL4/+; 20XUAS-6XGFP/+; (C, D) *yw; nompC-GAL4/+; 20XUAS-GFP/+; (E, F) *yw: nompC-GAL4/+; UAS-mCD8-GFP/+*. Arrows indicate cell bodies in all panels. Arrowheads indicate axons in panels (A), (C), and (E), and dendrites in panels (B), (D), and (F). All images were collected with a 63X water immersion objective using identical confocal settings. Axons and dendrites are brighter and more easily observed with the 20XUAS-6XGFP reporter than with the 20XUAS-GFP or UAS-mCD8GFP reporters. Scale bar 50 μ m.**

Figure S4 Examples of three-dimensional images used for quantitation in Figure 4. Imarus software was used to render three-dimensional images from which volume was quantitated. Genotypes: (A, B) *yw; nompC-GAL4/+; 20XUAS-6XGFP/+*; (C, D) *yw; nompC-GAL4/+; 20XUAS-GFP/+*; (E, F) *yw; nompC-GAL4/+; UAS-mCD8-GFP/+*. (B, D, and F) are rotated images of (A, C, and E). Volume quantitation strongly correlates with fluorescence intensity.

Figure S5 Pair-wise comparison of non-fluorescent visibility of the hexameric mCherry reporters with existing alternatives in the larval ventral nerve cord. (A) *yw; n-syb-GAL4/+; 20XUAS-6XmCherry-HA/+*; (B) *yw; n-syb-GAL4/+; 10XUAS-mCD8RFP/+*; (C) *yw; n-syb-GAL4/+; 20XUAS-6XmCherry-HA/20XUAS-6XmCherry-HA*; (D) *yw; n-syb-GAL4/+; 10XUAS-mCD8RFP/10XUAS-mCD8RFP*; (E) *yw; n-syb-QF2/+; 10XQUAS-6XmCherry-HA/+*; *yw; n-syb-QF2/+; 5XQUAS-mtdTomato-3XHA/+*; (G) *yw; n-syb-QF2/+; 20XQUAS-6XmCherry-HA/20XUAS-6XmCherry-HA*; (H) *yw* control. Third instar larva homozygous for *5XQUAS-mtdTomato-3XHA* in combination with *n-syb-QF2* were not obtained.

Figure S6 The hexameric GFP and mCherry proteins are visible with the naked eye in larva and adults when ubiquitously expressed. (A) *yw* control larva; (B, E) *yw; alpha-tubulin-QF2w/+; 10XQUAS-6XGFP/+*; (C, F) *yw; alpha-tubulin-QF2w/+; 10XQUAS-6XmCherry-HA/+*; (D) wildtype *Canton-S* control adult.