NATF (Native and Tissue-Specific Fluorescence): A Strategy for Bright, Tissue-Specific GFP Labeling of Native Proteins in Caenorhabditis elegans

Siwei He,*† Andrea Cuentas-Condori,† and David M. Miller, III*††

*Program in Neuroscience, Vanderbilt University, Nashville, Tennessee and †Department of Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, Tennessee 37240

ORCID IDs: 0000-0002-4847-0031 (A.C.-C.); 0000-0001-9048-873X (D.M.M.)

ABSTRACT GFP labeling by genome editing can reveal the authentic location of a native protein, but is frequently hampered by weak GFP signals and broad expression across a range of tissues that may obscure cell-specific localization. To overcome these problems, we engineered a Native And Tissue-specific Fluorescence (NATF) strategy that combines genome editing and split-GFP to yield bright, cell-specific protein labeling. We use clustered regularly interspaced short palindromic repeats CRISPR/Cas9 to insert a tandem array of seven copies of the GFP11 β-strand (gfp11,7) at the genomic locus of each target protein. The resultant gfp11,7 knock-in strain is then crossed with separate reporter lines that express the complementing split-GFP fragment (gfp1-10) in specific cell types, thus affording tissue-specific labeling of the target protein at its native level. We show that NATF reveals the otherwise undetectable intracellular location of the immunoglobulin protein OIG-1 and demarcates the receptor auxiliary protein LEV-10 at cell-specific synaptic domains in the Caenorhabditis elegans nervous system.

KEYWORDS C. elegans; cell specificity; genome editing; GFP; NATF; native expression; protein localization

RELIABLE localization of a given protein can provide useful clues to its mechanism of action. One way to achieve this goal is to label the protein of interest with tags, such as fluorescent proteins (e.g., GFP) (Chalfie 2009; Remington 2011) or small peptides (e.g., FLAG or HA) (Terpe 2003). Because tagged proteins are typically expressed with heterologous promoters or from multicopy transgenic arrays, this approach can result in misleading signals due to overexpression (Praitis et al. 2001). This problem can be obviated by using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 for single-copy labeling of the native protein (Hsu et al. 2014; Dickinson et al. 2015), but this genome-editing strategy suffers from two additional limitations. First, the endogenous expression level of a target protein may be too low for detection. Second, the protein of interest may be expressed in several tissues, thus preventing a clear delineation of cell-specific localization. Previous efforts have focused on solving each of these problems separately. For example, strategies to enhance detection include the use of brighter fluorescent proteins (El Mouridi et al. 2017; Hostettler et al. 2017) and the development of the SunTag label, which uses a scaffold-like structure to recruit multiple copies of GFP (Tanenbaum et al. 2014). The “FLP-on” strategy uses cell-specific FLP drivers to activate GFP expression from CRISPR/Cas9-engineered FRT sites but may not yield a visible signal for low-expressing genes (Schwartz and Jorgensen 2016). Here, we describe an experimental approach, NATF (Native And Tissue-specific Fluorescence or “Native”), that exploits a combinatorial strategy to achieve both bright and cell-specific labeling of the protein of interest.

Our approach relies on the finding that the barrel-like GFP structure can be reconstituted by the spontaneous interaction of two separate GFP peptides derived from the highly stable GFP variant, superfolder GFP. The larger of these fragments is comprised of the first 10 β-strands (GFP1-10). Its smaller complement, a short, 16 amino acid sequence, contains the 11th β-strand (GFP11) (Cabantous et al. 2005). The reconstituted

SIWEE HE, ANDREA CUENTAS-CONDORI, AND DAVID M. MILLER, III

1Corresponding author: Department of Cell and Developmental Biology, Vanderbilt University Medical Center, Vanderbilt University School of Medicine, 3120 MRBIII, Nashville, TN 37232-8240. E-mail: david.miller@vanderbilt.edu

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split-GFP hybrid produces a fluorescent signal that is substantially brighter than weak background fluorescence arising from over-expression of the GFP1-10 fragment (Feng et al. 2017). Thus, to enhance the GFP signal, a target protein can be tagged with multiple copies of the short GFP11 peptide and then co-expressed with excess GFP1-10 (Kamiyama et al. 2016; Feng et al. 2017). In addition to labeling the native protein with smaller covalent tags, this combinatorial approach offers the further benefit of limiting the GFP signal to the specific cell type in which GFP1-10 is expressed (Figure 1A).

In this report, we describe a NATF toolbox that combines split-GFP and CRISPR technology for live-cell imaging of labeled C. elegans proteins expressed at native levels. With this approach, a GFP11 multicopy DNA array (GFP11x7) is inserted into the target gene. The resultant knock-in strain can then be crossed with separate reporter lines in which GFP1-10 is expressed in different cell types for tissue-specific visualization of the reconstituted NATF GFP (Figure 1A). We utilized this strategy for effective enhancement of an otherwise weak signal from single-copy labeling of a key protein (OIG-1) as well as the cell-specific resolution of a receptor accessory protein (LEV-10) at closely spaced but functionally distinct synapses in the C. elegans nervous system.

Materials and Methods

C. elegans strains

C. elegans strains were maintained at room temperature on NGM plates seeded with OP50 (Brenner 1974). Some strains were obtained from the Caenorhabditis Genetics Center (CGC). The N2 Bristol strain was used as the wild-type reference. Transgenic animals were generated using standard microinjection techniques (Evans 2006). Strains used in this study are described in Supplemental Material, Table S1.

Molecular biology

single-guide RNA/Cas9 plasmid design: A 200 bp DNA sequence that contained the desired cut site was submitted to the optimized CRISPR Design online tool (http://crispr.mit.edu/) to predict single-guide RNA (sgRNA) sequences. To enhance gene-editing efficiency, we selected a 5′N10GGNGG sequence (Farboud and Meyer 2015) as an sgRNA targeting site for both oig-1 and lev-10. For oig-1, 5′-GGAGAGAAAGAC GAAAATGG-3′ was inserted into pDD162 (#47549; Addgene), a plasmid that contains the sgRNA backbone and Cas9 expression system, using Q5 site-directed mutagenesis (New England Biolabs) to create the repair template. The corresponding protospacer adjacent motif (PAM) PAM sequence in the repair template was mutated from AGG to CCC using Q5 site-directed mutagenesis to produce the final plasmid, pSH30. Correct insertions and mutations were confirmed by sequencing.

To create the SEC repair template (pSH55) for the oig-1 GFP11x7 CRISPR knock-in, the GFP11x7 coding sequence was amplified from a previously published plasmid (#70224; Addgene) and inserted into pSH30 to replace the TagRFP sequence in In-Fusion cloning (Takara) with the following primers.

Fragment.FOR 221 5′-GGTTGAATATGCATGCCTGAGC ACCATGGTGCCCTT-3′.

Fragment.REV 222 5′-AAGATCGATTTCTCGGTGATACCG GCAGCAT-3′.

Vector.FOR 223 5′-GAGAATCTGACTGTTACATCCGGAAA GGTAA-3′.

Vector.REV 224 5′-CGCATTTATCACAAGCAGTTAAACCAT AAAAGTAGT-3′.

To create the GFP and GFP11x7 knock-in repair template for LEV-10, we used a two-step In-Fusion cloning method. Next, ~500 bp of DNA sequences upstream and downstream of the lev-10 stop codon were selected for flanking homology arms. DNA was amplified, and then sequentially cloned into pSH30 or pSH55 to replace the original oig-1 homology arms. The resultant plasmids were then used as templates for site-directed mutagenesis to create the final repair template plasmid with sgRNA-binding sequences mutated, pSH84 (GFP knock-in), and pSH85 (GFP11x7 knock-in). The primers for these cloning steps were designed with a strategy similar that used for oig-1 as described above. Primer sequences are available on request.

GFP1-10 reporter plasmids: The DNA sequence of GFP1-10 was amplified from pCDNA1-1.GFP1-10 (#70219; Addgene) and cloned into pGHC (Prob-3:mCherry) using In-Fusion cloning to create pSP1 (Prob-3:mCherry) plasmid. The Dorsal D (DD) and Ventral D (VD) DD and VD γ-aminobutyric acid (GABA)ergic neuron-specific promoter Ptt-39, the cholinergic-specific

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promoter Pacr-2, and the muscle-specific Pmyo-3 promoter were amplified to replace the Prab-3 promoter in pSP1 to create pSH79 (Pptr-39::gfp1-10), pSH88 (Pacr-2::gfp1-10), pSH86 (Pmyo-3::gfp1-10), and pSH87 (Pfpl-13::gfp1-10). To create a secreted GFP1-10 construct, In-Fusion cloning was used to add the first 114 bp of the oig-1 sequence including the signal sequence (Philbrook et al. 2018) prior to the start codon of GFP1-10 in pSP1. The combined sequence was analyzed using the SignalP 4.1 Server to confirm that the predicted signal peptide was intact. The final plasmid, pSH69 (Prab-3::ssGFP1-10), was confirmed by sequencing.

Confocal microscopy and image processing
Fluorescent images were captured at room temperature using a Nikon (Garden City, NY) A1R confocal microscope. Nematodes were immobilized with 15 mM levamisole/0.05% tricaine on a 2% agarose pad in M9 buffer. All images for ACR-12::GFP fluorescence quantification were obtained with the same settings using a 40×/1.4 oil objective and Nyquist sampling. Constant laser power was used to compare the LEV-10::GFP fluorescence intensity to that of the NATF GFP signal produced by the combination of LEV-10::GFP11x7 with Pmyo-3::GFP1-10. Images in Figure 4 were three-dimensionally deconvolved with NIS-Elements with the automatic algorithm. For other images, ND2 files generated with NIS-Elements were imported into Fiji for analysis. Maximum intensity projections were generated by selecting stacks that had both ventral and dorsal signals. Line scans of dorsal and ventral cords (Figure S2, B–D), and of the nerve ring (Figure 3C), were adjusted by subtraction of background fluorescence measured from an adjacent region for comparison of fluorescence intensities between samples. To compare the stability of GFP signals in lev-10::gfp vs. lev-10::gfp11x7.

Figure 1 Robust, tissue-specific labeling of a target protein at its native expression level. (A) NATF. CRISPR/Cas9-mediated gene editing is used to label a protein of interest with seven copies of GFP11 (GFP11x7). Transgenic expression of GFP1-10 with cell-specific promoters results in a bright, stable NATF fluorescent signal from multiple, reconstituted GFP molecules in specific tissues. (B) NATF workflow. Worms are injected with sgRNA, repair template, and co-injection markers. gfp11x7 knock-in worms are recovered after heat shock-induced excision of positive-selection genes. Crossing the gfp11x7 knock-in with gfp1-10 reporter lines results in tissue-specific labeling of the target protein with a reconstituted NATF-GFP tag. CRISPR, clustered regularly interspaced short palindromic repeats; GABA, γ-aminobutyric acid; NATF, Native And Tissue-specific Fluorescence; sgRNA, single-guide RNA.
Pmyo-3::GFP1-10 strains, a region of interest (ROI) of the same size in each strain was bleached with a 405-nm laser for 15 sec at 50% laser power. Images of the ROI were collected and compared, before and after photobleaching. OIG-1::GFP111x7 and mCherry::OIG-1 strains were imaged using an A1R Nikon laser confocal to obtain intensity profiles (Figure 2A and Figure S3). Fiji was used to draw 15-µm long-line scans on the ventral cord and extract intensity profiles, which were exported to Excel. Fluorescent intensities were normalized to the maximum intensity value of each line scan. Intensities were plotted using Prism 6 software. Peaks exceeding a threshold of 75% of the normalized intensity for each sample were counted.

**AiryScan imaging**

Worms were mounted on 10% agarose pads and immobilized with 15 mM levamisole/0.05% tricaine dissolved in M9. A Zeiss ([Carl Zeiss], Thornwood, NY) LSM880 microscope equipped with an AiryScan detector and a 63×/1.40 Plan-Apochromat oil objective lens was used to acquire superresolution images of the DD neuron (Figure 4E). Images were acquired as a Z-stack (0.19 µm/step), spanning the total volume of the DD neuron and submitted for AiryScan image processing by ZEN software.

**Statistical analysis**

For all experiments, sample numbers were n > 10. The Student’s t-test was used for comparison between two groups. P < 0.05 was considered significant. Prism 6 was used for statistical analysis.

**Immunoblotting**

OIG-1 CRISPR and transgenic overexpression strains were cultured on NGM plates seeded with OP50. Mixed-stage worms were collected into a 1.5-ml tube by washing them off NGM plates with M9 buffer and pelleted by centrifugation. Next, 30 µl of 2× SDS-PAGE protein sample buffer was added to a 50 µl pellet of each genotype and heated to 90°C for 15 min. Samples were centrifuged at 8000 rpm for 5 min to remove debris (Miller et al. 1983). Then, 30 µl of supernatant from each genotype was loaded on a 10% protein gel to run at 110 V per gel for 30 min. The gels were stained with Coomassie Blue R250 and destained in water. The bands were visualized under UV light. The bands were excised from the gel and sent for sequenced (data not shown).

**Data availability**

All reagents and C. elegans strains described in this work are available on request. Plasmids will be deposited at Addgene and C. elegans strains submitted to the CGC stock center. Supplemental figures have been uploaded at the Genetics Society of America Figshare portal. Supplemental material available at [https://doi.org/10.25386/genetics.7898075](https://doi.org/10.25386/genetics.7898075).

**Results**

**Tool box and strategy for NATF GFP labeling**

We used a previously described CRISPR/Cas9 system for genome editing in C. elegans (Dickinson et al. 2015). In this approach, homology arms flank a self-excising cassette that carries positive selection markers (sqt-1) for the identification of transgenic worms (“rollers”) and drug resistance (hygR) for the detection of CRISPR/Cas9-induced integrants. A brief heat-shock treatment induces excision of the marker cassette to restore wild-type movement (“nonroller”) (Figure 1B). For split-GFP experiments, we replaced the fluorescent protein sequence in the original repair template plasmid with a gfp111x7 insert (Kamiyama et al. 2016). Homology arms of ~500 bp were used for the two genes targeted (oig-1 and lev-10) in this study (Figure S1A). We also constructed separate plasmids for expressing GFP1-10 in specific cell types including body muscles, all neurons, cholinergic neurons, and GABAergic neurons (Figure 1B and Figure S1B). In these lines, the GFP1-10 transgenes are carried as extrachromosomal arrays that are maintained by selecting for a pharyngeal co-injection marker (Pmyo-2::mCherry) (Figure 1B). Cell-specific drivers are flanked with multiple cloning sites to facilitate the construction of plasmids for GFP1-10 expression in other tissues (Figure S1B). gfp111x7 knock-in strains can be confirmed within 2 weeks of the initial injection and then crossed with GFP1-10-expressing lines for characterization (Figure 1B).

**NATF GFP labeling reveals the intracellular localization of OIG-1 in GABAergic motor neurons**

oig-1 encodes a soluble protein with a single immunoglobulin domain (Figure 2B) that is temporally regulated in GABAergic motor neurons to antagonize a synaptic remodeling program; in oig-1 mutants, a postsynaptic acetylcholine receptor (ACHr) containing the AChR subunit, ACR-12::GFP, is ectopically relocated from dorsal to ventral GABAergic neuron processes. OIG-1 is secreted when overexpressed from multicopy transgenic arrays to produce bright puncta adjacent to clusters of ACR-12::GFP (Figure 2, A and B) (He et al. 2015; Howel et al. 2015). To ask if OIG-1 is also secreted when expressed from the native locus, we used CRISPR/Cas9 to engineer a single-copy knock-in of the TagRFP together with a 3XFLAG epitope tag (Figure 2B and Figure S2, A–C). We used immunoblotting to confirm expression of TagRFP::3XFLAG::OIG-1 (Figure 2C) but failed to detect TagRFP expression in vivo either by TagRFP fluorescence (Figure 2, D–G) or by immunostaining against the 3XFLAG epitope (data not shown). To produce a potentially brighter signal, we created a gfp111x7::oig-1 knock-in (Figure 2B) with a sgRNA that targeted the same 5’-N9GGGNGG site used for the TagRFP insert (Farboud and Meyer 2015). This strategy was designed to enhance a potential fluorescent signal by attaching seven copies of the GFP11 peptide to the OIG-1 N-terminus (Kamiyama et al. 2016). Successful knock-in of gfp111x7 was confirmed by sequencing (data not shown).
We have previously shown that ACR-12::GFP in VD-class GABAergic motor neurons mislocalizes to the ventral side in oig-1 mutants, thereby resulting in an asymmetric ACR-12::GFP signal that is brighter in the ventral vs. dorsal nerve cords (Figure S2) (He et al. 2015). In contrast, in the wildtype, ACR-12::GFP puncta are evenly distributed between dorsal and ventral nerve cords. This symmetry is maintained in the gfp11x7::oig-1 strain, thus arguing that the GFP11x7 adduct does not significantly disrupt OIG-1 function (Figure S2, C and D). We then crossed the gfp11x7::oig-1 knock-in with a pan-neural Prab-3::gfp1-10 transgenic line. Consistent with our previous findings, the OIG-1 NATF GFP signal can be detected in head neurons, and in both dorsal and ventral nerve cords (Figure 2H-K) (He et al. 2015). Colocalization of OIG-1 NATF GFP with the nuclear-localized pan-neural marker Prab-3::NLS::mCherry confirmed OIG-1 expression in neurons (Figure 2L). As an independent strategy to validate OIG-1 expression in GABA neurons, we crossed the gfp11x7::oig-1 line with Pttr-39::gfp1-10, which is selectively expressed in DD- and VD-class GABAergic motor neurons (Cinar et al. 2005).
this case, the OIG-1 NATF GFP signal is limited to VD neurons with either weak or undetectable expression in DD neurons in L4 larvae (Figure 2M). This finding confirms previous results obtained with a Polg-1::gfp transcriptional reporter that was expressed in VD, but not DD, neurons after the L2 larval stage (He et al. 2015). Because the GFP1-10 peptide is expressed intracellularly in these strains, the NATF GFP signal likely derives from cytoplasmic OIG-1. Notably, the OIG-1 NATF GFP signal is visible throughout VD neuron soma and neurites (Figure 2, H–M), and does not show the distinctive highly punctate appearance of OIG-1 when overexpressed from a multicopy array (Figure 2A) (Figure S3) (He et al. 2015; Howell et al., 2015). To test for potential secretion of OIG-1 from the native locus, the gfp11,7::oig-1 knock-in was crossed with a transgenic line in which the GFP1-10 peptide is secreted from neurons (Prab-3::ss::gfp1-10). However, this experiment did not produce a detectable extracellular NATF signal nor GFP fluorescence in coelomocytes in the body cavity, which normally function as macrophage-like cells and thus can be used to detect secreted protein markers (Figure S4A) (Fares and Greenwald 2001). Notably, overexpression of mCherry::OIG-1 from an extrachromosomal array does label coelomocytes (He et al. 2015; Howell et al. 2015). As a positive control, we showed that the secreted form of GFP1-10 in the Prab-3::ss::gfp1-10 strain is functional because it robustly labels a GFP11 peptide fused to the extracellular domain of the synaptic membrane protein NLG-1 (Feinberg et al. 2008) (Figure S4, B–D). As negative controls, we showed that neither GFP11,7 nor GFP1-10 by themselves produce visible GFP fluorescence (Figure S2, E and F). Although undetectably low levels of secreted OIG-1 could be produced by this experiment, our overall results are consistent with the hypothesis that OIG-1 is not secreted when expressed at the native level but localizes intracellularly (S. He, A. Cuentas Condori, D. Miller, unpublished data). For example, genetic disruption of the OIG-1 signal peptide blocks OIG-1 secretion but does not disrupt oig-1 function in vivo (He et al. 2015). Our finding that OIG is intracellularly localized depended on the use of the NATF strategy to reveal low levels of native OIG-1 expression and thereby circumvent artificial extracellular localization due to OIG-1 overexpression from multicopy arrays (He et al. 2015).

**NATF GFP labeling reveals discrete locations for the transmembrane domain protein LEV-10 in different cell types**

Having shown that NATF could detect a soluble protein (OIG-1), we next targeted LEV-10, a CUB domain transmembrane protein that clusters AChRs at postsynaptic sites in body muscles (Gally et al. 2004). First, we created a CRISPR/Cas9 knock-in line in which a single copy of GFP was fused to the intracellular C-terminus of LEV-10 (see Figure 4A). We detected LEV-10::GFP in both ventral and dorsal nerve cords, as predicted for a protein that localizes to body muscle synapses (Gally et al. 2004). LEV-10::GFP puncta were also detected in the head region where motor neurons synapse with body muscles on the outside surface of the nerve ring (White et al. 1986; Von Stetina et al. 2006) (Figure 3A). For NATF GFP labeling of body muscle synapses, we generated a lev-10::gfp11,7 knock-in and crossed it with a muscle-specific line expressing GFP1-10 (Pmyo-3::gfp1-10) from an extrachromosomal array. The LEV-10 muscle-specific NATF GFP signal in the head region and axial nerve cords (Figure 3B) mimics that of the single-copy lev-10::gfp knock-in (Figure 3A), but is noticeably brighter. We quantified the GFP signal for each marker at the nerve ring muscle synapses to confirm that the LEV-10 NATF fluorescence is brighter (around three times) than the GFP signal from the lev-10::gfp single-copy insertion, as predicted from measurements of single-copy vs. multicopy split-GFP expressed in cultured cells (Figure 3C) (Kamiyama et al. 2016). In addition to determining that the lev-10::gfp11,7 array yields a stronger signal than that of the single-copy lev-10::GFP insert, we also showed that NATF GFP is substantially more resistant to photobleaching, as previously demonstrated for reconstituted split-GFP from measurements in vitro (Kamiyama et al. 2016) (Figure 3D).

In addition to expression in muscle, our independent studies have shown that LEV-10 is also expressed in ventral cord neurons where it colocalizes with AChRs at postsynaptic sites in GABAergic motor neurons (S. He, A. Cuentas Condori, D. Miller, unpublished data). In the motor neuron circuit, cholinergic motor neurons form dyadic synapses that innervate closely spaced postsynaptic domains in body muscle and GABA neurons (see Figure 4G) (White et al. 1986). Both of these postsynaptic regions in the ventral nerve cord region should be labeled in the lev-10::gfp knock-in and, thus, cannot be unambiguously identified (Figure 3A). To resolve this problem, we crossed the lev-10::gfp11,7 knock-in with transgenic lines that express GFP1-10 in either body muscles (Pmyo-3::gfp1-10), or in DD and VD GABAergic motor neurons (Pptr-39::gfp1-10). NATF GFP puncta can be readily detected in both cases (Figure 4, C and D), but are brighter in muscles than in GABAergic neurons (data not shown). Expression of a TagRFP-labeled AChR subunit UNC-29 (Gally et al. 2004) in muscle confirms colocalization of UNC-29::TagRFP with LEV-10 NATF GFP reconstituted in muscle. (Figure 4C). Expression of GFP1-10 in DD and VD neurons produces LEV-10 NATF GFP puncta that overlap with a cytoplasmic GABA neuron mCherry marker (Punc-47::mCherry), as predicted for the LEV-10 protein that localizes to GABA neuron synapses (Figure 4D). To confirm the postsynaptic location of LEV-10 in GABA neurons, we used a DD-specific construct (Pfp-13::gfp1-10) to generate a LEV-10 NATF GFP signal. In this case, superresolution imaging resolves distinct LEV-10 NATF GFP puncta at the tips of postsynaptic spine-like projections that have been recently described in the ventral processes of mature DD neurons (Figure 4E) (Philbrook et al. 2018). Notably, we have also observed that the AChR marker, ACR-12::GFP, is positioned in the same distal location in DD dendritic spines and that these spines are aligned with presynaptic cholinergic vesicles (Cuestas-Condori et al. 2019) (Philbrook et al. 2018). In addition to resolving
LEV-10 localization at distinct postsynaptic locations in muscle vs. GABA neurons, we also used a cholinergic motor neuron driver (\textit{Pacr-2}\textasciitilde\textit{gfp1-10}) to detect a separate LEV-10 NATF signal in ventral cord cholinergic neurons. In this case, LEV-10 NATF GFP is diffuse (Figure 4F) and asymmetrically localized to the ventral, but not dorsal, nerve cord (data not shown), a labeling pattern that closely resembles the perisynaptic position of the AChR subunit ACR-12::GFP in cholinergic motor neurons (Petrash et al. 2013). Because LEV-10 is expressed at its native level and retains its AChR clustering function (data not shown) when fused to the GFP11X7 adduct, it seems likely that each of the three distinct, cell-specific LEV-10 NATF signals (i.e., muscle, GABA neurons, and cholinergic neurons) marks authentic subcellular locations for the endogenously expressed LEV-10 protein.

Discussion

We have shown that NATF offers a robust strategy for producing bright, cell-specific signals for the \textit{C. elegans} proteins OIG-1 and LEV-10 expressed from their native genomic loci. These results suggest that NATF should be especially useful for marking connections in the compact \textit{C. elegans} nervous system, where most synapses are located in the densely packed nerve ring and axial nerve cords (White et al. 1986).

For example, GFP-tagging of a core presynaptic protein (e.g., RAB-3) by conventional CRISPR/Cas9 editing should mark synapses throughout the nervous system. In contrast, labeling with the NATF strategy should result in a bright, photostable GFP signal that is limited to the presynaptic domains of specific neurons. Although our results have determined that fusion with the GFP11X7 peptide does not result in detectable disruption of the in vivo function of either OIG-1 (Figure S2, B–D) or LEV-10 (Figure 4C and S. (S. He, A. Cuentas Condori, D. Miller, unpublished data), other proteins may be less tolerant. In that event, smaller adducts with fewer copies of GFP11 could be attempted. In that case, GFP signal augmentation will be diminished but tissue-specific labeling is still possible (Noma et al. 2017). Because the GFP11X7 insert is stably integrated at the native locus and is thus limiting, the complementing GFP1-10 peptide can be provided from multicopy transgenic arrays without risk of inducing overexpression artifacts. Thus, a given GFP11X7 split GFP insert can be rapidly tested with multiple tissue-specific GFP1-10 transgenic lines,

Figure 3 lev-10::gfp11x7 yields a stronger NATF GFP signal than the single-copy lev-10::gfp knock-in at synapses in neurons and muscle cells. (A) Confocal image showing localization of LEV-10::GFP in a single-copy GFP knock-in at the native lev-10 gene (lev-10::gfp). LEV-10::GFP puncta are visible at the nerve ring (arrow), and in ventral and dorsal nerve cords (arrowheads). (B) Confocal image of the LEV-10 NATF GFP signal at body muscle synapses arising from the combination of the lev-10::gfp11x7 knock-in with \textit{Pmyo-3}\textasciitilde\textit{gfp1-10}. NATF GFP (arrow) is detected at neuromuscular synapses near the nerve ring. Bar, 20 \textmu m. Insets (right) shows rotated views of anterior regions of images on left to depict nerve ring labeling. Asterisks mark gut autofluorescence. (C) LEV-10 NATF GFP at body muscle synapses in the nerve ring labeled with lev-10::gfp11x7 is significantly brighter (around three times) (3450 ± 441) than the single-copy lev-10::gfp knock-in (1280 ± 184). \(P < 0.001\), \(N = 15\), Student’s t-test. Error bars are SD. (D) The LEV-10 NATF GFP signal at body muscle synapses in the nerve ring labeled with lev-10::gfp11x7 is significantly more stable (0.75 ± 0.15) to photobleaching than the single-copy lev-10::gfp knock-in in the nerve ring (0.45 ± 0.12), \(N = 10\), \(P < 0.001\), Student’s t-test. See Materials and Methods. Error bars are SD. AU, arbitrary units; NATF, Native And Tissue-specific Fluorescence.
which can be readily generated using conventional methods. A similar combinatorial approach should also be useful for tissue-specific protein labeling in other model organisms (Kelliher et al. 2018). We note that NATF can be modified to reduce weak background fluorescence from the GFP1-10 fragment (Feng et al. 2017), and for multicolor split-GFP imaging with cyan (CFP) and yellow (YFP) GFP variants, or with the sfmCherry marker (Kamiyama et al. 2016; Feng et al. 2017).

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