Two-Color GFP Expression System for *C. elegans*

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ABSTRACT

We describe the use of modified versions of the Aequora victoria *green fluorescent protein (GFP) to simultaneously follow the expression and distribution of two different proteins in the nematode,* Caenorhabditis elegans*. A cyan-colored GFP derivative, designated CFP, contains amino acid (aa) substitutions Y66W, N146I, M153T and V163A relative to the original GFP sequence and is similar to the previously reported "W7" form. A yellow-shifted GFP derivative, designated YFP, contains aa substitutions S65G, V68A, S72A and T203Y and is similar to the previously described "10C" variant. Coding regions for CFP and YFP were constructed in the context of a high-activity* C. elegans *expression system. Previously characterized promoters and localization signals have been used to express CFP and YFP in* C. elegans*. Filter sets designed to distinguish YFP and CFP fluorescence spectra allowed visualization of the two distinct forms of GFP in neurons and in muscle cells. A series of expression vectors carrying CFP and YFP have been constructed and are being made available to the scientific community.*

INTRODUCTION

The ability to observe a population of molecules in living cells using the green fluorescent protein (GFP) has led to major advances in our understanding of many different biological questions (1,3,11,14,19). Recently, the utility of this technology has been enhanced by the development of GFP variants with altered fluorescence spectra. These different colored GFPs should prove to be very useful for marking distinct protein populations or cellular organelles for double- or multiple-labeling experiments (7,16,20).

For a multiple-labeling system to be generally useful in fluorescence microscopy, a number of criteria must be fulfilled. First, the different GFP forms must be sufficiently bright to be visible under standard epi-fluorescence illumination. Second, the fluorescence signal must be sufficiently stable to allow observation and documentation over extended times (several seconds to several minutes). Third, the spectra of the different GFP forms must be sufficiently different that they can be unambiguously distinguished using barrier filters.

Although a blue-shifted variant of GFP (BFP) can be spectrally resolved from GFP, it has a low quantum yield and is not photostable, which limits its usefulness (4,20). In contrast, more recently developed GFP mutants with cyan-shifted (CFP) and yellow-shifted (YFP) emission spectra are brighter and more stable than BFP (4,7,16). In addition, CFP and YFP exhibit well-separated emission and excitation spectra.

Here, we describe expression vectors and sets of barrier filters that can be used for CFP and YFP double-labeling experiments in *Caenorhabditis elegans*. Two recent publications have demonstrated the utility of CFP and YFP for dual-labeling in the wide-field microscope (4,21). In these studies, CFP and YFP are selectively excited through discrete band-pass filters, but common dichroic and emission barrier filters are used to capture both signals. This configuration avoids the registration shift that occurs with the use of separate dichroics and is therefore optimal for certain applications such as time-lapse imaging or high-resolution co-localization studies. This arrangement, however, effectively excludes most of the CFP emission spectrum. In addition, the use of a common emission filter produces spectrally overlapping signals from CFP and YFP, which therefore must be collected in separate channels and pseudo-colored before merging. We have used separate dichroic and emission filters to capture a substantially greater fraction of the CFP signal, with negligible cross talk between CFP and YFP, and also to produce direct two-color images that can be easily discerned by eye. Thus, the filter sets that we describe provide a useful alternative for cases in which registration shift is either not a limiting factor or can be easily corrected.

MATERIALS AND METHODS

Fluorescence Spectra

For in vitro analysis, CFP (Y66W, N146I, M153T and V163A) and YFP (S65G, V68A, S72A and T203Y) genes were subcloned into the pRSETA vector (6×His tag). Bacterially expressed CFP and YFP proteins were purified over a Ni-NTA Spin Column

Table 1. GFP Expression Plasmids

(*i*) **Plasmids with the *myo-3* promoter, simian virus 40 (SV40) nuclear localization signal and *unc-54* 3′ end** (body-wall muscle expression, incomplete nuclear localization with substantial signal in the cytoplasm):

myo-3::NLS::CFP::*unc-54* 3′ is L4663 (pPD133.48)

myo-3::NLS::YFP::*unc-54* 3′ is L4643 (pPD132.112)

(ii) **Plasmids with the *myo-3* promoter driving SV40NLS::GFP-lacZ** (exclusive nuclear localization—the addition of the bulky β-galactosidase (β-gal) to the nuclear localization signal (NLS)-tagged-GFP apparently enhances retention of the signal in the nucleus):

myo-3::NLS::CFP-lacZ::*unc-54* 3′ is L4660 (pPD133.45)

myo-3::NLS::YFP-lacZ::*unc-54* 3′ is L4671 (pPD133.63)

(*iii*) **Plasmids with the *myo-3* promoter, concatemerized (4×) SV40 NLS and *unc-54* 3′ end** (nucleolar localization). This reporter might produce cytotoxicity in some cells (A. Fire, unpublished data):

myo-3::4xNLS::CFP::*unc-54* 3′ is L4686 (pPD133.91)

myo-3::4xNLS::YFP::*unc-54* 3′ is L4687 (pPD133.97)

(*iv*) **Plasmids with the *myo-3* promoter, mitochondrial localization signal (9) and *unc-54* 3′ end** (mitochondrial localization):

myo-3::Mt::CFP::*unc-54* 3′ is L4665 (pPD133.54)

myo-3::Mt::YFP::*unc-54* 3′ is L4667 (pPD133.60)

(*v*) **Plasmids with the *myo-3* promoter driving GFP::lacZ chimera** (cytoplasmic localization):

myo-3::CFP-lacZ::*unc-54* 3′ is L4682 (pPD133.82)

myo-3::YFP-lacZ::*unc-54* 3′ is L4683 (pPD133.86)

These plasmids can serve as sources of spectrally distinct GFP versions with a variety of localization signals. For simplicity, all of these plasmids contain the *myo-3* promoter, which can easily be replaced by any promoter of choice.

(Qiagen, Valencia, CA, USA). Absorption and emission spectra were acquired as previously described (18).

Plasmids

All of the GFP expression plasmids described in Table 1 include an intron-punctuated GFP coding region, which produces substantially higher levels of expression than the unmodified coding region (5). Details of vector construction and complete sequences can be downloaded from our electronic archive (**http://www.ciwemb.edu**).

All of these plasmids can serve as a convenient source of the CFP and YFP coding regions, allowing introduction of these sequences into any existing GFP construct. Convenient restriction sites located upstream of the mutations that alter GFP fluorescence in these CFP and YFP variants include *Kpn*I, *Age*I and *Nco*I. Downstream sites include *Ava*II and *Eco*RI for both CFP and YFP, and *Mfe*I for CFP only.

Expression Plasmids Used to Create Images

unc-4::CFP (pDH1) is expressed in DA and VA motor neurons in the ventral nerve cord (13). pDH1 was created by replacing an *Age*I-*Mun*I restriction fragment of GFP in plasmid pNE-1 (17) with the corresponding region of the CFP plasmid, pPD115.46 (described at Web site: **http://www.ciwemb.edu**).

del-1::YFP (pDH2) is expressed in

VB motor neurons beginning in L2 larval stage and in VA motor neurons beginning later, in L3 larvae (22) (J. Ross and D. Miller, unpublished data). pDH2 was created by replacing an *Nco*I-*Eco*R1 restriction fragment of GFP in plasmid pJR6gfp (Winnier et al., unpublished) with the corresponding region from the YFP plasmid, pEYFP (CLONTECH, Laboratories, Palo Alto, CA, USA). Details of these plasmid constructions are available upon request.

The muscle-specific *unc-54* promoter in the vector pPD116.06 [*unc-54*::NLS::CFP-lacZ::*unc-54* 3′ UTR] produces strong nuclear localization due to the combined presence of the SV40 NLS and bulky β-gal protein. Plasmid pPD132.64 (*unc-54*::NLS::YFP::*unc-54* 3′ UTR) lacks the bulky lacZ moiety and results in predominantly cytoplasmic YFP staining.

Transgenic Lines

Transgenic lines were created by conventional methods (12). The *unc-54*-driven CPF and YFP expression vectors (pPD116.06, pPD132.64) (20 ng/μL each) were co-injected with the *rol*-6 dominant marker plasmid pRF4 (60 ng/μL) to produce a transgenic line expressing both CFP and YFP in muscle cells. The presence of the *unc-54* promoter results in a patchwork pattern of expression (i.e., mosaic), which we exploited to obtain clear images of brightly stained individual muscle cells. The *myo-3* promoter (15) is recommended for experiments aimed at producing a more uniform pattern of expression in muscle cells.

Transgenic lines arising from co-injection of the neuron-specific plasmids pDH1 and pDH2 did not express either CFP or YFP. Co-injection of alternative forms of these two expression vectors, in which lacZ is fused to the pDH1 promoter and GFP(S65C) is fused to the pDH2 promoter region, also did not result in detectable reporter gene expression in tranformed lines (unpublished data). This effect has not been observed for CFP and YFP plasmids bearing different promoter elements used in this work or in similar experiments performed by others (21). Thus, the promoter regions of these specific plasmids (pHD1 and pHD2) are apparently mutually inhibitory when incorporated into a common extrachromosomal array. To circumvent this unusual problem, we first created independent transgenic lines expressing either pDH1 or pDH2. pDH1 (50 ng/μL) and the *dpy-20*(+) co-selectable marker plasmid pMH86 (25 ng/μL) (2,13) were microinjected into *dpy-20*(*e1282*) mutant animals. pDH2 and the *lin-15*(+) co-selectable marker plasmid pJM23 (30 ng/μL) (8) were microinjected into *lin-15*(*n765*) animals. These strains were mated to produce a homozygous *dpy-20*(*e1282*); *lin-15*(*n765*) line (NC201) carrying both the pDH1 and pDH2 extrachromosomal arrays. NC201 was maintained by picking non-Dpy, non-Lin-15 animals with each generation.

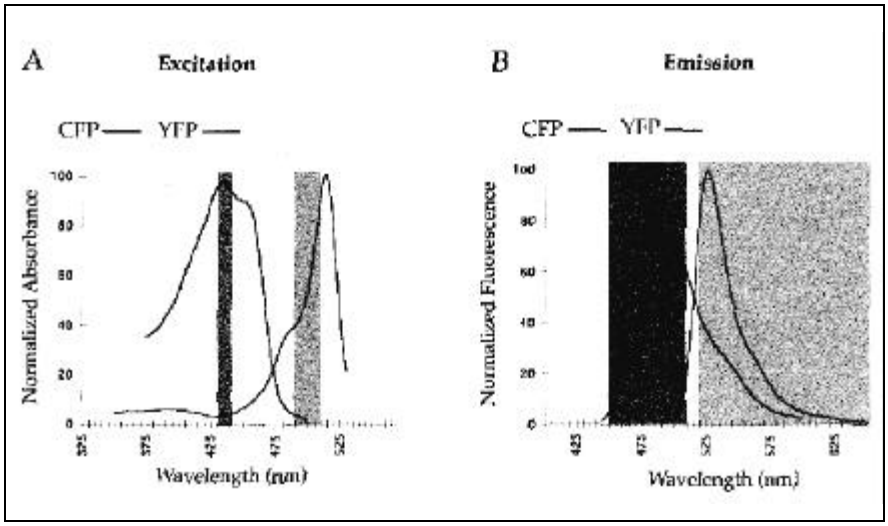

Figure 1. CFP and YFP excitation (A) and emission (B) spectra. Filled areas denote wavelengths and relative intensities of light passing through excitation and emission filter sets: CFP: excitation = 436 ± 5 nm, dichroic = 450 nm LP, emission = 480 ± 30 nm; YFP: excitation = 500 ± 10 nm, dichroic = 515 nm LP, emission = 520 nm LP.

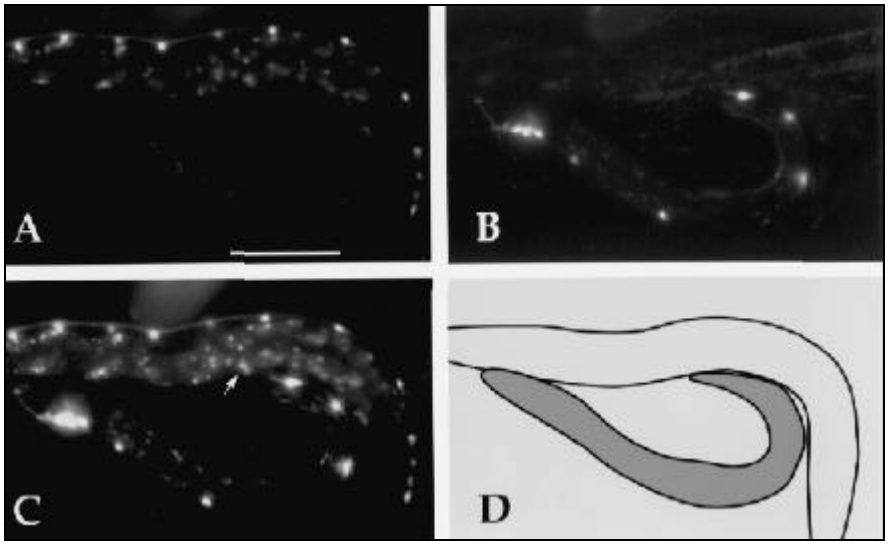

Figure 2. Discrete detection of CFP and YFP expression in *C. elegans* ventral nerve cord. (A) *del-1*::YFP expression in L2 larva visualized with YFP filter set. (B) *unc-4*::CFP expression in L1 larva visualized with CFP filter set. (C) Superposition of CFP and YFP images in Panels A and B demonstrates that the CFP filter set does not pass YFP fluorescence and that the YFP filter set does not pass CFP fluorescence. Arrowhead denotes autofluorescent gut granules. (D) Outlines of YFP-expressing nematode (green) and CFP-expressing (blue) animal shown in Panels A–C. Scale bar is 50 μm.

Fluorescence Filter Sets

The filter sets used in these experiments were purchased from Chroma Technology (Brattleboro, VT, USA) and include: (*i*) CFP, excitation, D436/10; beamsplitter, 450 dclp, emission, HQ 480/60. Although this configuration maximizes capture of the CFP emission peak, it also passes a low level of excitation light. Background illumination is reduced with the alternative filter set of excitation, HQ 436/20, and emission, HQ 480/40, but the signal tends to quench more rapidly with the wider band-pass excitation filter. (*ii*) YFP, excitation, HQ 500/20×; dichroic, Q515LP; emission HQ 520 long pass (Chroma YFP filter set No. 41029).

Evaluating Cross Talk Through CFP and YFP Filter Sets

Leakage of the CFP signal through the YFP filter set and cross talk of the YFP signal through the CFP cube were evaluated from images of CFP and YFP expressing neurons in transgenic lines derived from the pHD1 and pHD2 expression plasmids described above. Images were collected with a 10× Fluar, N.A. 0.5 or a 40× plan Neofluar, N.A. 1.3 objective lens on a Zeiss Axiophot® -2 (Carl Zeiss, Thornwood, NY, USA) and recorded in a Micromax camera with a KAF 1400 CCD (Princeton Instruments, Trenton, NJ, USA). To assess CFP leakage through the YFP filter set, an image of CFP neurons was obtained with the CFP filter set and compared to an image of the same field obtained with the YFP filter set. Reciprocal images were collected to assess YFP leakage through the CFP filters. Exposure times ranged from 0.05 s up to 3 s. Autofluorescent granules in the gut, which are visible with both filter sets, were used to align the paired images. The fluorescence intensity of corresponding regions in each image pair was obtained using NIH Image 1.61 (**http://rsb.info.nih.gov/nih-image/**) in an Apple Macintosh® G3/300 computer (Apple Computer, Cupertino, CA, USA). Estimates of background signal were obtained from adjacent regions. Even at exposure times that saturated pixels in the YFP channel with gut autofluorescence; there was no signal from CFP-positive neurons detectable over background (11 neurons from 5 animals). Pixels overlapping the same neuron in the CFP channel gave >10 000 photons over the adjacent background. YFP could be detected leaking into the CFP channel at about 0.1% of the level in the YFP channel (38 neurons in 5 animals). However, the exposure time required to detect this small YFP leak was 50-fold longer than the exposure time needed to collect a bright CFP image.

Microscopy

Images were collected with either a 63× Plan Apo, N.A. 1.4 or a 100× Plan Neofluor, N.A. 1.3 objective in a Zeiss Axioplan® microsope (Carl Zeiss) and recorded with a Cooled CCD Spot Camera (Diagnostic Instruments, Sterling Heights, MI, USA) or on Ektachrome 400 film (Eastman Kodak, Rochester, NY, USA). Comparable results were obtained in a Nikon E800 microscope (Nikon, Melville, NY, USA). For the charge-coupled device (CCD) camera images, CFP signal was collected with the Blue channel, and YFP fluorescence was detected with the combined Green and Red channels. Exposure times varied from 1–10 s. Tagged Image Format Files (TIFF) were imported into Adobe® Photoshop® (Adobe Systems, San Jose, CA, USA) in an Apple Macintosh G3/300 computer and superimposed to produce two-color images (Figure 4). Slide film was double-exposed (20–35 s) for both CFP and YFP (Figures 2 and 3) and digitized in a Nikon LS-1000 35-mm Slide Scanner (Nikon). Contrast and color balance were adjusted in Photoshop for the final images.

RESULTS AND DISCUSSION

The ideal fluorescence filter set maximizes signal from a given probe while minimizing leak-through from the excitation light and other fluorophores. As shown in Figure 1, the fluorescence spectra of CFP and YFP exhibit large shifts in both excitation and emission maxima (see Materials and Methods). We chose filter sets that exploit the differences in both excitation and emission between the two mutant proteins to obtain spectrally discrete signals from CFP and YFP. The 436/10 band-pass filter overlaps the excitation maximum of CFP (435 nm) but should result in minimal excitation of YFP (Figure 1A). The 480/60-nm band-pass emission filter captures approximately 70% of the CFP emission peak but passes very little of the YFP fluorescence (Figure 1B). It is possible to collect most of the CFP peak with a 460-nm, long-pass (LP) emission filter without significant YFP leak-through; however, this configuration appears to increase the relative intensity of background autofluorescence (data not shown). An alternative combination that also produces a bright CFP signal includes a 436/20 excitation filter coupled with a 480/40 emission filter (See Materials and Methods). The YFP filter set includes a 500/20 band-pass filter, which excites YFP but not CFP (Figure 1A), and a 520 LP filter that spans most of the YFP emission peak (Figure 1B). In this case, CFP fluorescence is effectively eliminated from the YFP channel by the 500/20-nm barrier filter, which prevents CFP excitation (Figure 1A).

The applicability of these filter sets was determined by direct observation of transgenic nematodes expressing CFP and YFP in motor neurons and in body-wall muscle cells. Although the fluorescence intensity of these probes in vivo was not quantitatively measured, we noted photographic exposure times (5–30 s) on film that are comparable to those of the F64L S65T and S65C variants of GFP (data not shown). Thus, these CFP and YFP probes are sufficiently bright and stable to be useful for in vivo labeling experiments in *C. elegans*.

The filter sets that we have selected are capable of producing spectrally discrete signals from either CFP or YFP. As shown in Figure 2, a nematode expressing YFP in ventral cord motor neurons is readily detected with the YFP barrier filter, but an adjacent CFP-positive animal is not visible unless viewed through the CFP filter. Similarly, we do not detect YFP fluorescence in a separate image of the same pair of nematodes visualized with the CFP filter set (Figure 2B). These observations were confirmed by direct, quantitative measurements that detected minimal

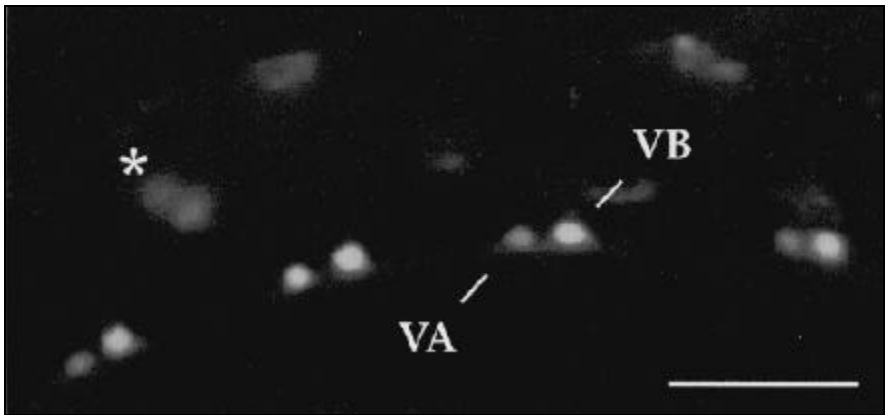

Figure 3. Color-coding motor neurons. *unc-4*::CFP (blue) is expressed in VA motor neurons and *del-1*::YFP (green) is expressed in VB motor neurons in this L2 stage larva. Asterisk marks gut autofluorescence. Scale bar is 20 μm.

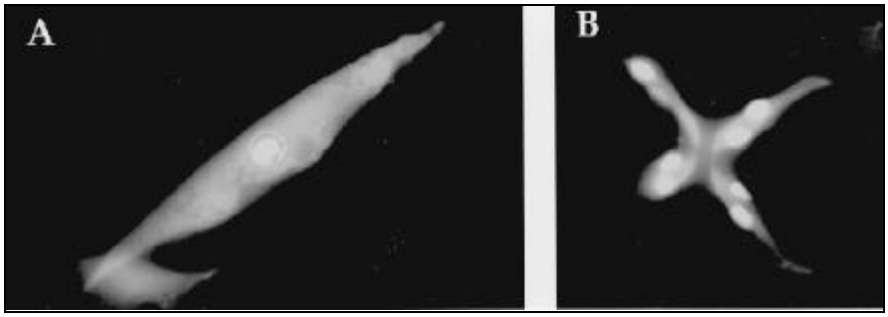

Figure 4. Detection of CFP and YFP signals in muscle cells. The *unc-54* promoter was used to drive nuclear-localized CFP (blue) and cytoplasmic YFP (green) in (A) a body-wall muscle cell and (B) vulval muscle cells.

cross talk through these filter sets (see Materials and Methods). This specific combination of filter sets offers the additional advantage of producing direct two-color images, which are easily discerned by eye in the microscope and in film and electronic recordings.

Separate classes of motor neurons in the *C. elegans* ventral nerve cord can be readily distinguished using a transgenic strain with cell-specific expression of CFP and YFP (Figure 3). In these images, the *unc-4* promoter drives expression of CFP in VA motor neurons (13), and the *del-1* promoter directs YFP expression to VB motor neurons (22) (J. Ross and D. Miller, unpublished data). Inclusion of a synthetic segment encoding the SV40 NLS peptide (6) in both of these constructs localizes most of the fluorescence signal to neuronal nuclei.

This technology should also be useful for simultaneous labeling of different intracellular compartments. To illustrate this point, we used the *unc-54* promoter (15) to drive expression of CFP and YFP in body-wall (Figure 4A) and vulval muscles (Figure 4B). The *unc-54*::CFP construct (pPD116.06) targets the nucleus, whereas the *unc-54*::YFP construct (pPD132.64) is designed to produce predominantly cytoplasmic staining (see Materials and Methods). Blue CFP-stained nuclei are distinctly visible in the background of green cytoplasmic YFP expression (Figure 4). Expression vectors that localize CFP or YFP to mitochondria or nucleoli have also been constructed (see Materials and Methods). In the future, it should be possible to label other intracellular compartments (e.g., cytoplasmic membranes, neuronal synapses, muscle fibers etc.) by fusing CFP or YFP to specific proteins that target these regions (10,21).

We have constructed a series of vectors that can be used to drive CFP and YFP expression in *C. elegans* neurons and muscle cells (see Materials and Methods). These vectors can be easily adapted to insert different promoters for expression in other cells. Alternatively, the CFP and YFP coding regions can be subcloned into existing GFP expression vectors.

All of these vectors are available to the scientific community. Detailed descriptions of these vectors are available (**www.ciwemb.edu**).

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REFERENCES

1. **Chalfie, M., Y. Tu, G. Euskirchen, W.W. Ward and D.C. Prasher**. 1994. Green fluorescent protein as a marker for gene expression. Science *263*:802-805.
2. **Clark, D. V., D.S. Suleman, K.A. Beckenbach, E.J. Gilchrist and D.L. Baillie.** 1995. Molecular cloning and characterization of the dpy-20 gene of *Caenorhabditis elegans*. Mol. Gen. Genet. *247*:367-378.
3. **Cubitt, A.B., R. Heim, S.R. Adams, A.E. Boyd, L.A. Gross and R.Y. Tsien.** 1995. Understanding, improving and using green fluorescent proteins. Trends Biochem. Sci. *20*:448-455.
4. **Ellenberg, J., J. Lippincott-Schwartz and J.F. Presley.** 1998. Two-color green fluorescent protein time-lapse imaging. BioTechniques *25*:838-846.
5. **Fire, A., J. Ahnn, W. Kelly, B. Harfe, S. Kostas, J. Hsieh, M. Hsu and S. Xu.** 1998. GFP applications in *C. elegans*, p. 153-168. *In* M. Chalfie and S. Kain (Eds.), GFP Strategies and Applications. John Wiley & Sons, New York.
6. **Fire, A., S.W. Harrison and D. Dixon.** 1990. A modular set of lacZ fusion vectors for studying gene expression in *Caenorhabditis elegans.* Gene *93*:189-198.
7. **Heim, R. and R.Y. Tsien.** 1996. Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. Curr. Biol. *6*:178-182.
8. **Huang, L.S., P. Tzou and P.W. Sternberg.** 1994. The *lin-15* locus encodes two negative regulators of *Caenorhabditis elegans* vulval development. Mol. Cell. Biol. *5*:395-411.
9. **Jaussi, R., B. Cotton, N. Juretic, P. Christen and D. Schumperli.** 1985. The primary structure of the precursor of chicken mitochondrial aspartate aminotransferase. Cloning and sequence analysis of cDNA. J. Biol. Chem. *260*:16060-16063.
10. **Jorgensen, E.M., E. Hartwieg, K. Schuske, M.L. Nonet, Y. Jin and H.R. Horvitz.** 1995. Defective recycling of synaptic vesicles in synaptotagmin mutants of *Caenorhabditis elegans*. Nature *378*:196-199.
11. **Llopis, J., J.M. McCaffery, A. Miyawaki, M.G. Farquhar and R.Y. Tsien.** 1998. Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins. Proc. Natl. Acad. Sci. USA *95*:6803-6808.
12. **Mello, C. and A. Fire.** 1995. DNA transformation, p. 451-482. *In* H.F. Epstein and D.C. Shakes (Eds.), Methods in Cell Biology, 48, *Caenorhabditis elegans*, Modern Biological Analysis of an Organism. Academic Press, New York.
13. **Miller III, D.M. and C.J. Niemeyer.** 1995. Expression of the *unc-4* homeoprotein in *Caenorhabditis elegans* motor neurons specifies presynaptic input. Development *121*:2877-2886.
14. **Miyawaki, A., J. Llopis, R. Heim, J.M. McCaffery, J.A. Adams, M. Ikura, and R.Y. Tsien.** 1997. Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. Nature *388*:882-887.
15. **Okkema, P.G., S.W. Harrison, V. Plunger, A. Aryana and A. Fire.** 1993. Sequence requirements for myosin gene expression and regulation in *Caenorhabditis elegans*. Genetics *135*:385-404.
16. **Ormo, M., A.B. Cubitt, K. Kallio, L.A. Gross, R.Y. Tsien and S.J. Remington.** 1996. Crystal structure of the *Aequorea victoria* green fluorescent protein. Science *273*:1392-1395.
17. **Pflugrad, A., J.Y.-J. Meir, T.M. Barnes and D.M. Miller III**. 1997. The Groucho-like transcription factor UNC-37 functions with the neural specificity gene *unc-4* to govern motor neuron identity in *C. elegans*. Development *124*:1699-1709.
18. **Piston, D.W., G.H. Patterson and S.M. Knobel.** 1998. Quantitative Imaging of the Green Fluorescent Protein (GFP), Chapter 3, p. 31-48. Methods in Cell Biology, Volume 58. Academic Press, New York.
19. **Prasher, D.C., V.K. Eckenrode, W.W. Ward, F.G. Prendergast and M.J. Cormier.** 1992. Primary structure of the *Aequorea victoria* green-fluorescent protein. Gene *111*:229-233.
20. **Rizzuto, R., M. Brini, F. De Giorgi, R. Rossi, R. Heim, R.Y. Tsien and T. Pozzan.** 1996. Double labeling of subcellular structures with organelle-targeted GFP mutants *in vivo*. Curr. Biol. *6*:183-188.
21. **Rongo, C., C.W. Whitfield, A. Rodal, S.K. Kim and J.M. Kaplan.** 1998. LIN-10 is a shared component of the polarized protein localization pathways in neurons and epithelia. Cell *94*:751-759.
22. **Tavernarakis, N., W. Shreffler, S. Wang and M. Driscoll.** 1997. *unc-8*, a DEG/ENaC family member, encodes a subunit of a candidate mechanically gated channel that modulates *C. elegans* locomotion. Neuron *18*:107-119.

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