CHAPTER 9

Expression and Imaging of Fluorescent Proteins in the *C. elegans* Gonad and Early Embryo

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Abstract

The *Caenorhabditis elegans* gonad and early embryo have recently emerged as an attractive metazoan model system for studying cell and developmental biology. The success of this system is attributable to the stereotypical architecture and reproducible cell divisions of the gonad/early embryo, coupled with penetrant RNAi-mediated protein depletion. These features have facilitated the development of visual assays with high spatiotemporal resolution to monitor specific subcellular processes. Assay development has relied heavily on the emergence of methods to circumvent germline silencing to allow the expression of transgenes encoding fluorescent fusion proteins. In this chapter, we discuss methods for the expression and imaging of fluorescent proteins in the *C. elegans* germline, including the design of transgenes for optimal expression, the generation of transgenic worm lines by ballistic bombardment, the construction of multimarker lines by mating, and methods for live imaging of the gonad and early embryo.

I. Introduction

A. The *Caenorhabditis elegans* Gonad and Early Embryo: A Model System for Cell and Developmental Biology

The *Caenorhabditis elegans* gonad and early embryo have recently emerged as powerful model systems for studying cell and developmental biology (for reviews see Hubbard and Greenstein, 2000; Hyman and Oegema, 2005). Each arm of the gonad in the adult *C. elegans* hermaphrodite is an assembly line of ~800 nuclei progressing through the various stages of meiotic prophase in an ordered fashion (Fig. 1). The distal region of the gonad is a syncytium of partially enclosed nuclei that share a common maternal cytoplasm. In the proximal region of the gonad, individual nuclei become packaged into distinct oocytes. Oocytes are fertilized as they pass through the spermatheca, which sits at the proximal tip of the gonad. Hermaphrodites produce sperm at an earlier stage of larval development, prior to the switch to oogenesis (for details on the spermatogenesis–oogenesis switch, see Kimble and Strome, 2005). As the oocytes are fertilized, they pass into the uterus, where the resulting embryos complete meiotic segregation of the oocyte-derived

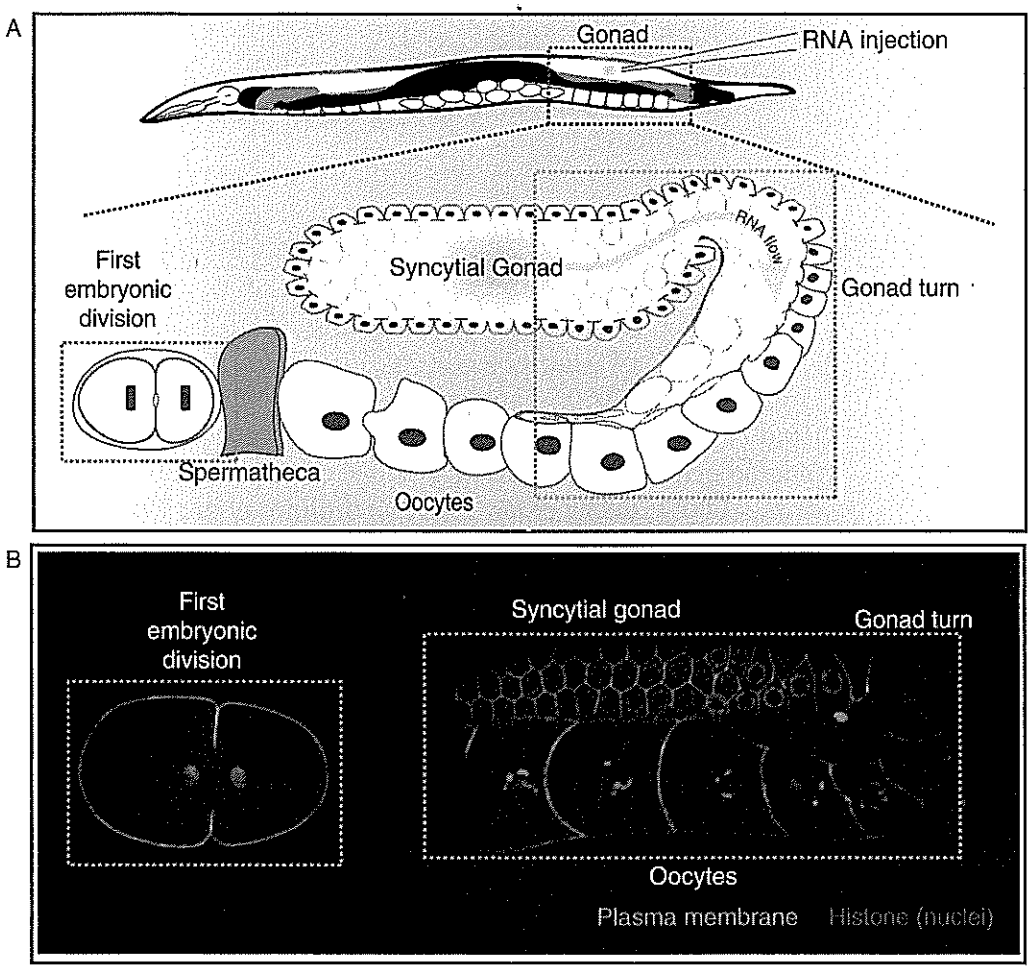


Fig. 1 The *C. elegans* gonad and early embryo. (A) The schematic shows one arm of the gonad in the adult *C. elegans* hermaphrodite to illustrate the assembly-line process that gives rise to oocytes and embryos. As they pass the turn in the gonad arm, individual compartments containing a meiotic nucleus are loaded with cytoplasm from the syncytial gonad and expand to form the developing oocytes. Oocytes are fertilized as they pass through the spermatheca, giving rise to early embryos. Introduction of dsRNA against a specific target rapidly catalyzes the destruction of the corresponding mRNA. The target protein present when the RNA is introduced is depleted by the continual packaging of the maternal cytoplasm into oocytes. (B) Live imaging of the gonad (right) and an early embryo (left) in a *C. elegans* strain expressing mCherry-histone and a GFP fusion with a PH domain (GFP-PH$^{PLC\delta1}$) to mark chromosomes and the plasma membrane, respectively (A. Audhya, Oegema Laboratory, unpublished strain). (See Plate no. 21 in the Color Plate Section.)

chromosomes (generating the oocyte pronucleus and two polar bodies), and subsequently undergo several rounds of mitotic cell division.

One of the primary advantages of the gonad/early embryo as a model system is efficient RNAi-mediated protein depletion, which facilitates quantitative analysis of the loss-of-function phenotypes for essential proteins. The reproducibility of protein depletion is due to the syncytial architecture of the gonad. Introduction

of dsRNA rapidly catalyzes the destruction of the corresponding mRNA in many different systems. However, depletion of preexisting protein is generally a slow process that depends on the half-life of the targeted protein. In contrast, in the *C. elegans* gonad, the protein present when the dsRNA is introduced is depleted by the continual packaging of maternal cytoplasm into oocytes. Since depletion depends on the rate of embryo production, the kinetics tend to be similar for different targets. By 36–48 h after introduction of the dsRNA, newly formed oocytes are typically >95% depleted of the target protein (reviewed in Oegema and Hyman, 2005). The reproducibility of RNAi-mediated protein depletion has led to a series of genome-wide screens (Fernandez *et al.*, 2005; Fraser *et al.*, 2000; Gonczy *et al.*, 2000; Kamath *et al.*, 2003; Maeda *et al.*, 2001; Piano *et al.*, 2000; Rual *et al.*, 2004; Simmer *et al.*, 2003; Sonnichsen *et al.*, 2005) that have complemented genetic approaches (for some examples, see Encalada *et al.*, 2000; Golden *et al.*, 2000; Gonczy *et al.*, 1999; Kemphues *et al.*, 1988; O'Connell *et al.*, 1998) to define the sets of genes required for embryo production and viability.

B. Quantitative Imaging-Based Assays Capitalize on the Rapid, Invariant Early Embryonic Cell Divisions

In addition to efficient RNAi-mediated protein depletion, the stereotypical architecture of the gonad, and the rapid and highly reproducible mitotic divisions of the early embryo facilitate the development of imaging-based methods to assess the consequences of molecular perturbations. The invariant nature of the first few mitotic divisions (Fig. 2) allows any parameter of interest (such as the fluorescence intensity of a localized protein, the size or the distance between subcellular structures, the number of microtubules touching the cortex, the extent of chromosome condensation, etc.) to be measured as a function of time with respect to a specific temporal landmark (such as the permeabilization of the nuclear envelope, the onset of chromosome segregation in anaphase, or the initiation of furrow ingression). The average value of the parameter measured in multiple embryos can then be plotted as a function of time to generate kinetic profiles that reveal behaviors not evident from qualitative viewing of individual time-lapse sequences. For example, quantitative analysis has provided detailed information on *pronuclear movement* (O'Connell *et al.*, 2000), *cortical flow* (Cheeks *et al.*, 2004; Hird and White, 1993; Munro *et al.*, 2004), *positioning of the spindle and anaphase spindle elongation* (Cheeseman *et al.*, 2004; Colombo *et al.*, 2003; Labbe *et al.*, 2004; Tsou *et al.*, 2002), *chromosome segregation and kinetochore-microtubule attachment* (Cheeseman *et al.*, 2004; Labbe *et al.*, 2004), and *chromosome condensation* (Maddox *et al.*, 2006).

The development of imaging-based assays has relied on the parallel emergence of methods to circumvent germline silencing, allowing for the expression of transgenes encoding fluorescent fusion proteins in the gonad and early embryo. Critical for this has been the development of vectors containing regulatory

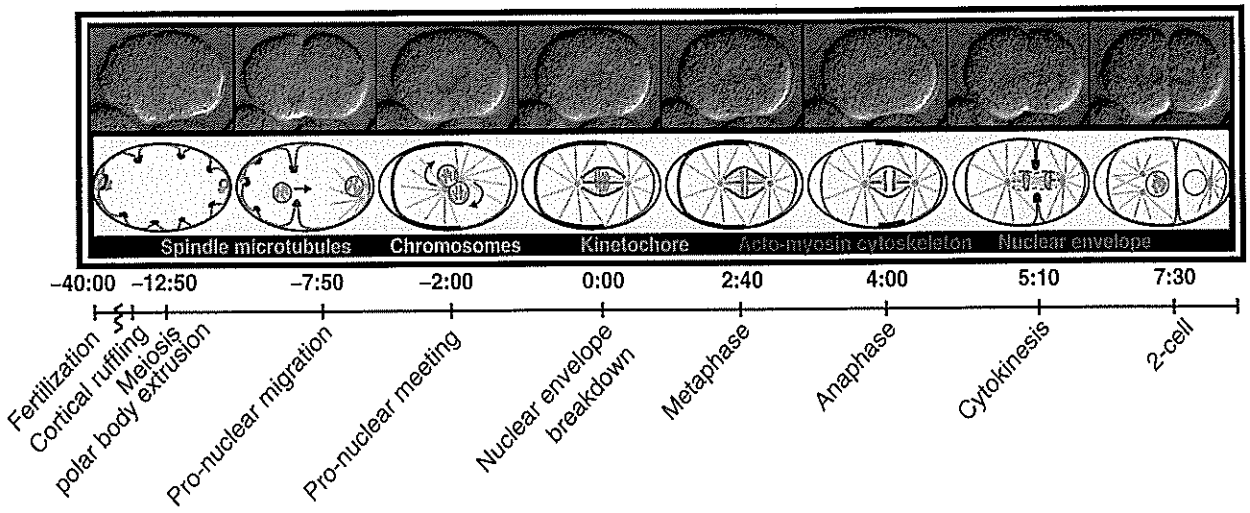


Fig. 2 Timeline of events in the *C. elegans* early embryo. Differential interference contrast images (top) and schematics (bottom) illustrate the timing of major events during the first mitotic division. Approximate times are in minutes:seconds with respect to nuclear envelope breakdown. Images are courtesy of Amy Maddox. Figure adapted from Oegema and Hyman (2005). (See Plate no. 22 in the Color Plate Section.)

sequences that direct germline expression of transgenes (Strome *et al.*, 2001) and the emergence of efficient ballistic bombardment techniques to integrate vectors into the genome at low-copy number (Praitis *et al.*, 2001; Wilm *et al.*, 1999).

II. Fluorescent Proteins in the *C. elegans* Gonad and Early Embryo

A. Fluorescent Proteins Commonly Used in *C. elegans*

To date, the majority of *C. elegans* work with fluorescent proteins has utilized an uncommon enhanced green fluorescent protein (GFP) variant in which serine 65, one of the three residues of the chromophore, is mutated to cysteine (S65C). GFP S65C is reported to have similar excitation and emission spectra to GFP S65T, the most commonly used enhanced variant due to its greater brightness, faster oxidation to final fluorescent product, slower photobleaching, and lack of photoisomerization when compared to wild-type GFP (Cubitt *et al.*, 1995; Heim and Tsien, 1996; Heim *et al.*, 1994). The common use of the unusual S65C variant in *C. elegans* is a historical consequence of its inclusion in the original vectors for *C. elegans* GFP expression, developed by Andrew Fire's group (Fire *et al.*, 1998). The decision to use S65C was based on its greater photostability *in vivo* in worms (A. Fire, personal communication). However, a systematic test comparing the properties of GFP S65C to GFP S65T in the *C. elegans* gonad and early embryo has not been performed.

In addition to GFP (S65C), the common color variants yellow fluorescent protein (YFP) (S65G, V68A, S72A, and T203Y) and cyan fluorescent protein (CFP) (Y66W, N146I, M153T, and V163A) have also been engineered for use in *C. elegans* (Miller *et al.*, 1999), and sequences encoding these fluorescent proteins have been incorporated into germline expression vectors (Franz *et al.*, 2005; Galy *et al.*, 2003).

Variants of the coral red fluorescent protein, DsRed, are emerging as the most popular choice as a dual color marker with GFP for live imaging. Due to its naturally tetrameric state, DsRed is poorly suited for *in vivo* studies (Matz *et al.*, 1999). Extensive mutagenesis generated a monomeric version from which a spectrum of fluorescent proteins, called the mFruits, has been developed (Campbell *et al.*, 2002; Shaner *et al.*, 2004, 2005). Of these, the most widely used is mCherry. Initial attempts to express mCherry in the *C. elegans* germline were unsuccessful; however, codon optimization and the inclusion of introns have overcome these problems (McNally *et al.*, 2006). Below we outline the strategy used to adapt mCherry as a means of providing guidelines for optimization of other fluorescent proteins for *C. elegans* germline expression.

B. Engineering New Fluorescent Proteins for Expression in the *C. elegans* Gonad/Early Embryo: The mCherry Experience

To reengineer mCherry for expression in *C. elegans*, we changed the codon usage and inserted artificial introns. Due to the variation in codon usage between species, optimal expression of a gene derived from one species in another typically requires "codon optimization." However, changing each of the codons for a specific amino acid to the most-favored codon is also not ideal; balance is important. Based on the analysis of numerous highly expressed genes, a favored codon usage value between 60 and 75% is predicted to yield optimal gene expression (Duret and Mouchiroud, 1999). We used this as a guideline in reengineering the mCherry coding sequence. As a second step, we introduced introns, which are known to stimulate expression in *C. elegans* (Fire *et al.*, 1990). We note, however, that although the reengineering of mCherry was successful, the contribution of each of these steps to the final outcome is unclear, since neither step was performed on its own. This point should be kept in mind when reading the mCherry reengineering procedure and using it to reengineer other fluorescent proteins.

1. Optimizing Codon Usage

For codon optimization of mCherry, we first listed all amino acids in the primary sequence noting their frequency and distribution. Then, based on the *C. elegans* codon usage table (see Fig. 3), we reassigned 70% of the codons to the optimal triplet for each amino acid in a random fashion. Next, we reassigned 5–10% of the remaining codons to the most poorly used triplet. The optimal and poorly used codons were well spaced to avoid prolonged stretches of poorly

used codons. Finally, we reassigned the residual 20% of codons to other triplets. After completing this process, we used web-based bioinformatic programs to check for five potentially adverse features in the designed sequence:

1. *Guanine-cytosine (GC) content bias*: The GC content of the entire *C. elegans* coding genome is ~43% and we checked to make sure that the GC content of the reencoded sequence approximated this value.

2. *Repetitive sequences*: Such sequences can lead to ribosome slippage and should be eliminated.

The codons for each amino acid are listed in order by frequency. Codons are color-coded to indicate whether their frequency is ***increased****, **decreased,*** or ***unaltered*** in highly-expressed genes.

ALA	ARG	ASN	ASP
GCU*	CGU*	AAC*	GAU
GCC*	AGA	AAU	GAC*
GCA	CGC*		
GCG	CGA		
	CGG		
	AGG		
CYS	**GLN**	**GLU**	**GLY**
UGC*	CAA	GAG*	GGA*
UGU	CAG*	GAA	GGU
			GGC
			GGG
HIS	**ILE**	**LEU**	**LYS**
CAC*	AUC*	CUC*	AAG*
CAU	AUU	CUU*	AAA
	AUA	UUG	
		CUG	
		UUA	
		CUA	
MET	**PRO**	**PHE**	**SER**
AUG*	CCA*	UUC*	UCC*
	CCG	UUU	UCU
	CCU		UCA
	CCC		UCG
			AGC
			AGU
STOP	**THR**	**TYR**	**VAL**
UAA*	ACC*	UAC*	GUC*
UAG	ACU	UAU	GUU
UGA	ACA		GUG
	ACG		GUA

Adapted from Duret and Mouchiroud (1999)

Fig. 3 Codon bias in *C. elegans*. Codons are listed by frequency of usage (in order of most common to least used) for expressed short proteins. Codons listed in green (*) are biased for usage in highly expressed sequences (more frequently used in highly expressed sequences when compared to low to nonexpressed sequences). Codons listed in red are biased against usage in highly expressed sequences. Codons listed in black are used at an equivalent frequency irrespective of expression level. This information was adapted from data presented in Duret and Mouchiroud (1999). (See Plate no. 23 in the Color Plate Section.)

3. *mRNA secondary structure*: Available programs such as the Vienna RNA package (http://www.tbi.univie.ac.at/~ivo/RNA/) are useful in predicting mRNA stability and structure. Incorporation of hairpin loops or other secondary structure elements that increase mRNA stability should be avoided.

4. *Cryptic splice sites*: The Alternative Splice Site Predictor (http://es.embnet.org/~mwang/assp.html) will identify potential cryptic splice sites. Such sites must be eliminated prior to the synthesis of the reencoded gene.

5. *tRNA steric hindrance*: Steric interactions between tRNAs have been shown to impact their ability to simultaneously occupy the two available ribosomal binding sites during translation (Smith and Yarus, 1989). Commercial programs, including DNA 2.0 (http://www.dnatwopointo.com/commerce/misc/opt.jsp), can be used to predict tRNA steric hindrance and eliminate it prior to finalizing the design of the reencoded gene.

2. Insertion of Introns

Incorporation of multiple introns has been shown to improve gene expression in *C. elegans* (Fire *et al.*, 1990). Stimulation by intronic sequences does not necessarily require the introns to be inserted into coding regions; inclusion within the 5′ or 3′ UTR can also increase gene expression by several folds. To prevent intramolecular recombination after transformation, use of identical introns is not recommended. Although a number of potential introns are available from the genome database, care must be exercised in choosing an appropriate sequence. Most importantly, introns with cryptic splice sites should be avoided. Although insertion of introns into a target gene is relatively straightforward, it requires several cloning steps. The use of unique blunt-end restriction sites is most convenient, allowing quick insertion of introns encoded by synthetic oligonucleotides. Alternatively, introns can be added to a target gene prior to its synthesis. The three artificial introns that were inserted into the coding sequence of mCherry are identical to those that were inserted into GFP (S65C) by Fire *et al.* (1998) [these sequences were: (1) gtaagtttaaacatatatatactaactaaccctgattatttaaattttcag, (2) gtaagtttaaacagttcggtac taactaaccatacatatttaaattttcag, (3) gtaagtttaaacatgattttactaactaactaatctgatttaaatttt cag].

Recent data indicate that introns in germline-expressed genes exhibit a high level of periodicity in their DNA structure, often showing a strong bias for AA/TT dinucleotides along one face of the DNA helix (Fire *et al.*, 2006). This bias is postulated to affect the association of intronic sequences with nucleosomes. Improper spacing of introns may affect their ability to form an appropriate DNA structure, and thereby compromise germline expression. However, further study is needed to examine this issue.

3. Other Considerations in Designing Transgenic Constructs

A final consideration is the position of the fluorescent tag with respect to the coding sequence. Potential protein modifications must be considered when incorporating the tag. For example, the N-terminus of myristoylated proteins should be left free to allow accessibility to N-myristoyltransferase. Also, although most fluorescent proteins encode flanking sequences both upstream and downstream of the fluorophore, an additional spacer is often useful to prevent protein misfolding.

III. Transgene Expression in the *C. elegans* Germline: Breaking the Silence

The efficiency of germline silencing has posed significant challenges to the expression of transgenes (Seydoux and Strome, 1999) encoding fluorescent fusion proteins. Although no strategy has fully circumvented this difficulty, it can be rendered manageable by the appropriate selection of regulatory sequences (Strome *et al.*, 2001) combined with the use of ballistic bombardment as the means of transgene delivery (Praitis *et al.*, 2001; Wilm *et al.*, 1999). In this section, we describe the most commonly used vectors and highlight important practical considerations when designing constructs for germline expression.

A. Promoter and 3′ UTR Choice

Although there are selected examples of successful germline expression under the control of endogenous regulatory sequences, the most commonly used strategy is to place coding regions under the control of the regulatory sequences for PIE-1, a transcriptional repressor that is highly expressed in the germline. This strategy, developed by Seydoux and coworkers, has proven remarkably effective in minimizing the germline silencing of transgenes (Strome *et al.*, 2001). In addition to the *pie-1* promoter, the *pie-1* 3′ UTR is also important to avoid silencing. In our experience, proteins expressed under control of the *pie-1* regulatory sequences are typically present at 5–50% of the levels of the endogenous protein when analyzed by Western blotting. The fact that this is consistently true, despite wide variation in the normal expression levels of the proteins tagged, likely reflects the importance of coding region sequence structure and potentially codon usage, in controlling protein levels. Functional rescue of mutants has been observed for many *pie-1* controlled transgenes. In some cases, increased levels of the transgenic protein are seen in the mutant background, possibly reflecting selection against silencing when transgene expression becomes essential for survival.

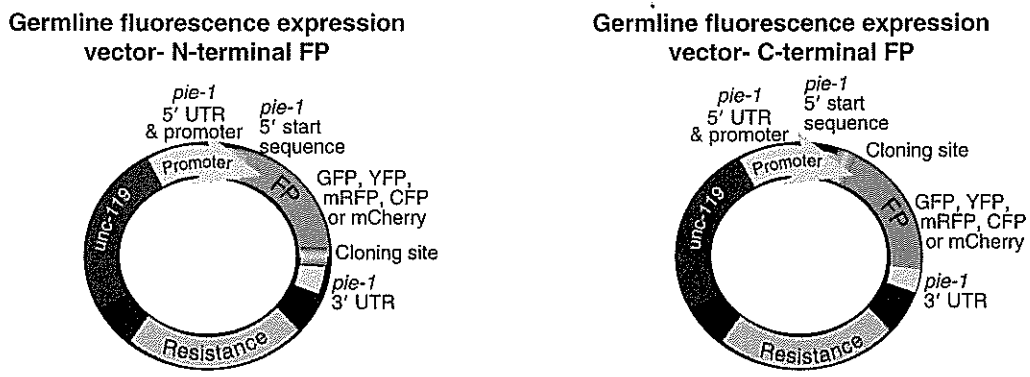
B. Currently Available Vectors for Expression of Fluorescent Proteins in the Germline

The currently used vectors for germline expression are based on pPD95.75, a vector generated in Andrew Fire's laboratory containing a version of GFP modified for expression in *C. elegans* (available from Addgene, www.addgene.org/pgvec1/f=c&identifier=1494&cmd=findpl). Geraldine Seydoux's laboratory modified this vector to place the GFP fusion under the control of the *pie-1* regulatory sequences, resulting in pJH4.52 (Strome *et al.*, 2001). pJH4.52 was designed to be linearized, mixed with a second linearized plasmid containing a dominant or selectable marker and an excess of cut genomic DNA, and injected into the gonad to generate stably transmitted complex extrachromosomal arrays (Kelly *et al.*, 1997), a popular technique that works well for expression in somatic tissues. Since arrays suffer from silencing, due to the presence of repetitive sequences, pJH4.52 was further modified in Judith Austin's laboratory to create pAZ132 (Praitis *et al.*, 1991), a vector designed for direct integration by ballistic bombardment. pAZ132 contains a cassette that drives the expression of UNC-119, the most popular selectable transformation marker for bombardments. In addition, it contains a cassette that directs the expression of protein fusions with GFP at their N-terminus under the control of the *pie-1* regulatory regions. Insertion of a tandem protein purification tag between the GFP and the target gene was performed in Arshad Desai's laboratory (pIC26) to allow biochemical isolation of the GFP fusion and its associated proteins [for detailed protocols, see Cheeseman and Desai (2005), Cheeseman *et al.* (2004)]. A derivative of pAZ132 that fuses GFP to the C-terminus of the target protein, pAZ132-C, has also recently been engineered in Tony Hyman's laboratory. In addition, the Hyman laboratory has generated a series of vectors that eliminate extraneous restriction sites, incorporate a multiple cloning site, reduce the size of these relatively large vectors by introducing a shortened region of *unc-119*, and carry distinct resistance and fluorescence markers. These smaller constructs offer greater convenience for cloning; however, occasionally incomplete rescue of the Unc phenotype makes the identification of moving worms, following bombardment, more challenging. Vectors incorporating other fluorescent proteins, such as mCherry (pAA64 and pAA65), YFP (pPAG4 and pAZ-YFP), and CFP (pUP9 and pAZ-CFP) have also been made in the laboratories of Oegema, Hyman, and Askjaer. A list of the available vectors for germline expression of fusions with fluorescent proteins can be found in Fig. 4.

IV. Constructing Fluorescent Worm Lines

A. Integration of Constructs by Ballistic Bombardment

Ballistic bombardment, in which pressurized helium is used to "bombard" small transgene-coated gold particles into worm tissue at high speeds, is the method of choice for introduction of germline expression constructs into *C. elegans*. Bombardment has proven vastly superior to the more traditional method of injecting



	Plasmid name	Promoter	Fluorescent protein	Location	Resistance	Selection marker	Additional features	Source
Parental vectors	pPD95.75		GFP					Fire lab
	pJH4.52	*pie-1*	GFP	N-terminus	Amp			Seydoux lab
	TH317-NoTag-AMP	*pie-1*	No tag	N-terminus	Amp	*unc-119*	short *unc-119*	A. Pozniakovsky; Hyman lab
	TH318-NoTag-CM	*pie-1*	No tag	N-terminus	Cm	*unc-119*	short *unc-119*	A. Pozniakovsky; Hyman lab
	TH319-NoTag-KAN	*pie-1*	No tag	N-terminus	Kan	*unc-119*	short *unc-119*	A. Pozniakovsky; Hyman lab
GFP vectors	pAZ132 (N-GFP)	*pie-1*	GFP	N-terminus	Amp	*unc-119*		Fire lab
	pIC26 (N-LAP)	*pie-1*	GFP	N-terminus	Amp	*unc-119*	LAP	I Cheeseman; Desai lab
	TH304-GFP (C) AMP	*pie-1*	GFP	C-terminus	Amp	*unc-119*	short *unc-119*, MCS	A. Pozniakovsky; Hyman lab
	TH303-GFP (N) AMP	*pie-1*	GFP	N-terminus	Amp	*unc-119*	short *unc-119*, MCS	A. Pozniakovsky; Hyman lab
	TH305-GFP (C) CM	*pie-1*	GFP	C-terminus	Cm	*unc-119*	short *unc-119*, MCS	A. Pozniakovsky; Hyman lab
	TH314-LAP (N) AMP	*pie-1*	GFP	N-terminus	Amp	*unc-119*	short *unc-119*, MCS, LAP	A. Pozniakovsky; Hyman lab
	TH315-LAP(N)GW-AMP	*pie-1*	GFP	N-terminus	Amp/Cm	*unc-119*	short *unc-119*, MCS, GateWay, LAP	A. Pozniakovsky; Hyman lab
mCherry vectors	pAA65(LAP)	*pie-1*	mCherry	N-terminus	Amp	*unc-119*	LAP, codon optim.	A. Audhya; Oegema lab
	pAA64	*pie-1*	mCherry	N-terminus	Amp	*unc-119*	codon optim.	A. Audhya; Oegema lab
	TH313-Cherry (C) AMP	*pie-1*	mCherry	C-terminus	Amp	*unc-119*	short *unc-119*, MCS codon optim.	A. Pozniakovsky; Hyman lab
	TH312-Cherry (N) AMP	*pie-1*	mCherry	N-terminus	Amp	*unc-119*	short *unc-119*, MCS codon optim.	A. Pozniakovsky; Hyman lab
mRFP vectors	TH309-mRFP (N) AMP	*pie-1*	mRFP	N-terminus	Amp	*unc-119*	short *unc-119*, MCS	A. Pozniakovsky; Hyman lab
	TH310-mRFP (C) AMP	*pie-1*	mRFP	C-terminus	Amp	*unc-119*	short *unc-119*, MCS	A. Pozniakovsky; Hyman lab
	TH311-mRFP (N) CM	*pie-1*	mRFP	N-terminus	Cm	*unc-119*	short *unc-119*, MCS	A. Pozniakovsky; Hyman lab
CFP vector	TH306-CFP(N) AMP	*pie-1*	CFP	N-terminus	Amp	*unc-119*	short *unc-119*, MCS	A. Pozniakovsky; Hyman lab
YFP vectors	TH307-YFP(N) AMP	*pie-1*	YFP	N-terminus	Amp	*unc-119*	short *unc-119*, MCS	A. Pozniakovsky; Hyman lab
	TH308-3xYFP(N) AMP	*pie-1*	Triple YFP	N-terminus	Cm	*unc-119*	short *unc-119*, MCS	A. Pozniakovsky; Hyman lab

Fig. 4 Vectors for the expression of fluorescent fusion constructs in the *C. elegans* gonad and early embryo. Schematics illustrate the common features for expression vectors that fuse a fluorescent protein to the N-terminus (left) or C-terminus (right) of the target protein. Each contains the 5′ UTR, promoter, and 5′ initial coding sequence for PIE-1, followed by fluorescent protein and 3′ *pie-1* UTR. The plasmids also contain a resistance marker for growth in *E. coli* and a selection marker (*unc-119)* for integration into the *C. elegans* genome. The list outlines features of currently available vectors, including promoter, fluorescent protein, location of fluorescent protein (N- or C-terminus), type of resistance marker (Amp, Cm, Kan), *C. elegans* selection marker (*unc-119)*, specific features of each plasmid, and the source of each vector. Specialized features include (1) the addition of multiple cloning sites, (2) the presence of a localization and affinity purification tag, (2) optimized fluorescent proteins for expression in *C. elegans* (intron incorporation and codon bias), (3) GateWay compatible cloning vector, and (4) truncated versions of *unc-119* (to reduce overall plasmid size). Some key parental vectors are also listed. (See Plate no. 24 in the Color Plate Section.)

the construct into the gonad to generate heritable and repetitive extrachromosomal arrays. Although expression from extrachromosomal arrays is relatively straightforward in somatic tissues, germline expression from even the best arrays is silenced within a few generations (Kelly and Fire, 1998; Seydoux and Strome, 1999).

Bombardment techniques were adapted to *C. elegans* to avoid problems associated with extrachromosomal arrays (Jackstadt *et al.*, 1999; Praitis *et al.*, 1991; Wilm *et al.*, 1999). The most widely used method for the generation of low-copy number transformants was developed by Praitis *et al.* (2001). This approach reduces the likelihood of generating large extrachromosomal arrays because a single bombarded bead can carry only a limited amount of DNA into a cell.

Bombardments are typically performed in the DP38 strain harboring the *unc-119(ed3)* mutation. These worms are unable to move and importantly also fail to undergo the starvation-induced developmental transition to the dauer stage. Integration of the *unc-119* genomic region, which is present in the bombarded plasmid, allows transformed worms to produce moving progeny that can survive starvation. Untransformed worms on the same plate remain uncoordinated and die. This strong selection is necessary since large quantities of worms must be bombarded to generate the relatively rare desired integration events.

1. Standard Small-Scale Bombardment Protocol

The protocol discussed below is for bombardments on standard small (60 mm diameter) plates using the Bio-Rad PDS-1000 apparatus (see Fig. 5) that requires a vacuum source (a pump or a good house vacuum line) and a helium tank. For each construct, we recommend seeding 10–12 large (100 mm) plates with DP38 worms to generate sufficient quantities of worms for 10 small bombardment plates. Bombardments can also be performed on large (100 mm) plates using the hepta-adaptor attachment (Bio-Rad, Hercules, CA; see details below).

a. Preparation of the Worms

1. Evenly seed DP38 (*unc-119(ed3)*; Maduro and Pilgrim, 1995) worms from two slightly starved 60-mm plates onto 10–12 large (100 mm diameter) peptone/c600 plates. This can be done by dividing each starved out DP38 plates into six chunks with a scalpel and dabbing each chunk onto a peptone/c600 plate. Peptone plates and the fast growing C600 strain of *Escherichia coli* are used to increase the number of worms per plate. The plates should be maintained at 20°C until they begin to starve out (about 1 week).

2. Using sterile M9 and a 10-ml pipette, wash the worms off the peptone plates by pipeting up and down. Transfer the washed worms from all 10 to 12 plates into a 50-ml conical tube. After allowing the worms to settle to the bottom of the tube, remove excess supernatant, leaving ~1–2 ml of liquid/worms in the bottom of each conical tube.

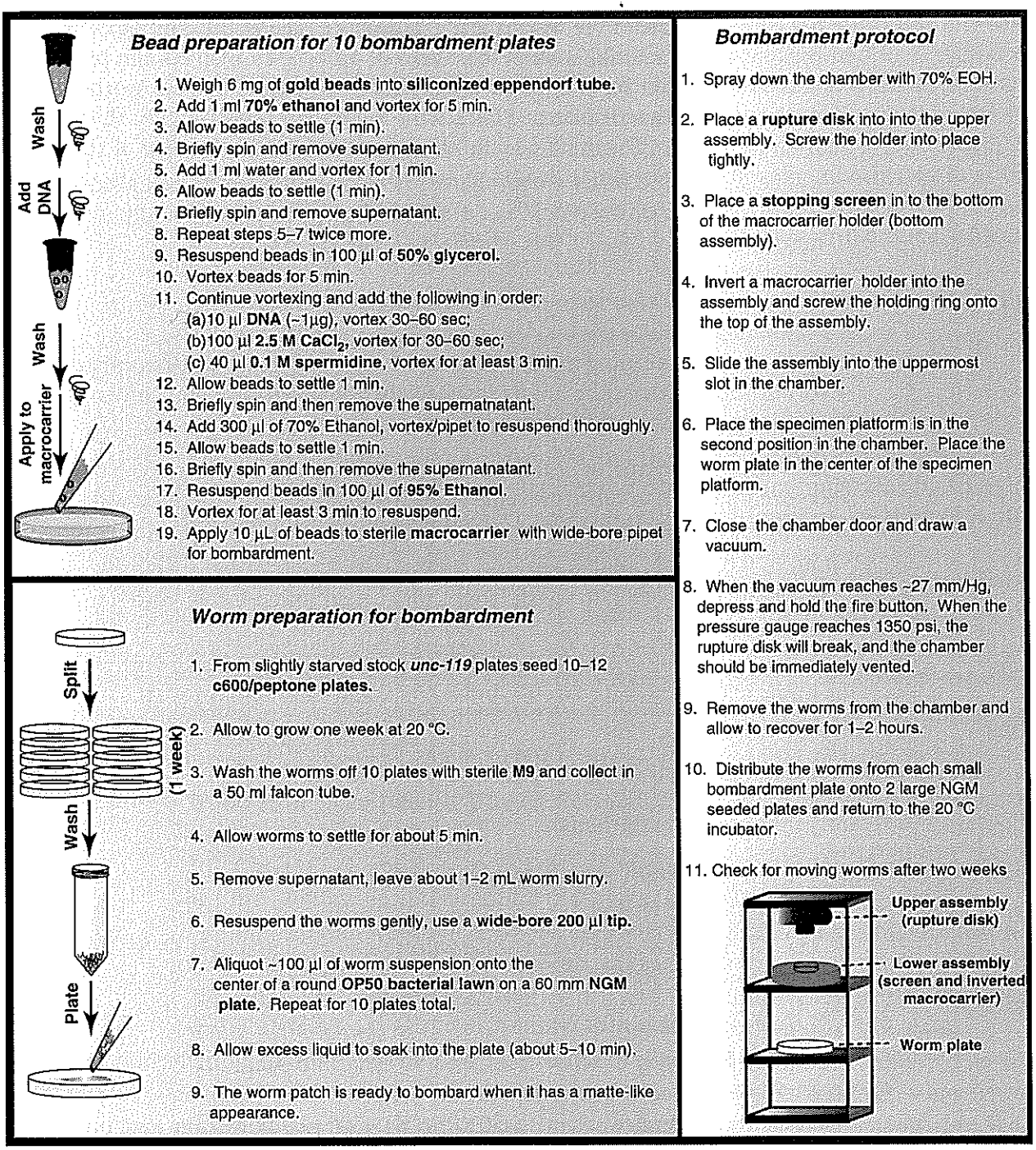


Fig. 5 Standard small-scale bombardment protocol. This figure details the preparation of gold beads and worms for a small-scale bombardment (one or two constructs). A protocol for bombarding the worms is also included (see chapter text for more detailed information). (See Plate no. 25 in the Color Plate Section.)

3. Gently resuspend the worms using wide-bore pipette tips and pipet 100 µl of worms onto the bacterial lawn in the center of an NGM/OP50 60-mm plate. Each conical tube should have enough worms for ~10 plates. Excess worms can be divided between the 10 plates. Plates with a circular lawn roughly the size of a quarter are the best for this since the worms will spread to the edge of the lawn.

4. The worms are ready to bombard when the excess liquid has absorbed into the plate and the worm patch has a matte-like appearance (usually after about 10 min).

b. Preparation of the Beads

The instructions below are for the preparation of beads for 10 bombardments. A vortex mixer with a tube holder (such as the TurboMix attachment from Vortex Genie) is very useful for this procedure.

1. Weigh out 6 mg of gold beads (Bio-Rad 1 µm beads #1652263) into a siliconized microfuge tube (prevents beads from sticking to the tube walls).

2. Add 1 ml of 70% ethanol (EtOH) and vortex the beads continuously for 5 min.

3. Allow the beads to settle for 1 min, then briefly spin and remove the supernatant.

4. Wash the beads three times with 1 ml water; after each wash vortex the beads for 1 min, allow the beads to settle, briefly spin, and carefully remove the supernatant for each wash.

5. Resuspend the beads in 100 µl of 50% glycerol and vortex the beads for 5 min.

6. Continue to vortex, pausing only briefly to add the following in order (vortex for 1 min between additions):

   i. 10 µl of DNA (about 1 µg)
   ii. 100 µl 2.5 M $CaCl_2$
   iii. 40 µl 0.1 M spermidine tissue culture grade, Sigma S-4139, St. Louis, MO, store at −20°C

7. Vortex the beads for 3 min.

8. Allow the beads to settle to the bottom for 1 min, spin briefly, and remove the supernatant.

9. Perform a final wash with 70% EtOH (300 µl), vortexing and pipeting to thoroughly resuspend the beads. Allow them to settle out, spin briefly, and remove the supernatant.

10. Resuspend the beads in 100 µl 95% EtOH, vortexing and pipeting to thoroughly resuspend the beads. Using a wide-bore pipette tip, pipet 10 µl of bead slurry onto the center of each sterile bombardment macrocarrier (10 in total).

Note: The DNA-coupled beads have a tendency to clump. To avoid this problem, proceed expeditiously through steps 6–10 (without stopping) and load the beads onto the macrocarriers as soon as possible after the final resuspension in 100% EtOH. Macrocarriers are small plastic discs that are mounted in a metal holder for the bombardment (see Bio-Rad manual for more details). It is convenient to mount them in the holders in advance and sterilize them by autoclaving in a foil-covered beaker. Alternatively, the macrocarriers can be sterilized by dipping in 70% EtOH, placing them in the holders, and putting them in the hood with the ultraviolet light on in an open petri dish.

11. Place the macrocarriers in an open petri dish inside a desiccator chamber to facilitate drying. *Use the prepared carriers within 2 h.*

c. Bombarding the Worms

1. Some contamination is inevitable, but this can be minimized by taking some precautions. Before beginning, sterilize the bombardment apparatus by spraying down the inside of the chamber and the upper and lower assemblies with 70% EtOH.

2. Using forceps, dip a rupture disk (Bio-Rad, #1652330) into 70% isopropanol and place it flush into the base of the upper assembly disk holder unit. Screw the disk holder into place and tighten with a torque wrench.

3. Next, place a stopping screen (Bio-Rad, #1652336) at the base of the lower assembly unit, then invert a macrocarrier holder on top of the screen and screw the holding ring into place.

4. Slide the lower unit into the top slot in the bombardment chamber. Place an OP50/NGM plate (seeded packed worms) on the specimen platform below the lower assembly unit.

5. Close the chamber door and draw a vacuum of 28 mm/Hg. Depress and hold the "fire" button until the rupture disk pops (around 1350 psi).

6. Immediately vent the chamber and remove the plate of worms. Repeat this procedure for the remaining macrocarriers/worm plates.

7. Allow the worms to recover for 1 h and then transfer the worms from each 60 mm plate to two or three 10 cm NGM/OP50 plates using an M9 wash.

8. Place all plates at 20°C and wait ~3 generations (about 12 days). The vast majority of the worms will remain uncoordinated and die; however, some worms will be successfully transformed. Since three generations have passed, plates on which a worm was transformed will be clearly identifiable by the presence of multiple moving siblings (progeny of the original transformed worm).

9. Single three young moving worms from each plate with movers onto individual small plates and screen for expression of the fluorescent transgene in their progeny. Usually, the three progeny will yield similar results, since they are most

likely siblings—but it is also possible for more than one worm to have been transformed per plate.

The progeny of these worms can be visually screened under the microscope for the incorporation of the fluorescently tagged gene of interest to the appropriate location (i.e., germline). If after several generations, you notice that one third of the progeny are uncoordinated, it is likely that the bombardment generated an obligate heterozygous transgenic line. This can occur, for example, if the transgene inserts into an essential gene. While these lines can be quite useful, they cannot be readily used to generate double (crossed) fluorescent lines. If all progeny are coordinated for several generations, then you have successfully generated a homozygous transgenic line. These lines can easily be mated with other fluorescent strains to develop multimarker and/or multicolor worm lines (see below).

2. Adapting the Protocol for Large-Scale Bombardments

For large-scale bombardments, larger quantities of DP38 can be obtained by culturing them in liquid. If bombardments are ongoing in the laboratory, a synchronous culture can be initiated and maintained for multiple generations. In the protocol below, we describe a two-step procedure to initiate a synchronous liquid culture. In the *first* step, an asynchronous liquid culture is started from plates and grown until the majority of worms are adults. The adults are isolated and their embryos obtained by treatment with a basic bleach solution. After hatching the embryos overnight without food to synchronize the population as starved L1s, the worms are reseeded into a *second* synchronous round of liquid culture. After a synchronous culture of DP38s is started, it can be maintained indefinitely by harvesting two third of the worms at each generation and obtaining embryos from the remaining one third to seed the subsequent round of culture.

a. Step 1: Starting an Asynchronous Liquid Culture of DP38s

Approximately 6 days prior to starting the culture: Seed 10–15 large (100 mm) OP50 plates with 30 clean worms each; incubate the worms at 20°C for ~6 days (or until worms are just starved).

Two days prior to starting the culture: Seed a 50-ml overnight bacterial culture with OP50–1 in LB plus 50 μg/ml streptomycin.

One day prior to starting the culture: Use the 50 ml culture to seed 2 × 1 liter overnight cultures of OP50–1 in LB plus 50 μg/ml streptomycin. This will be enough bacteria to start 2 × 500 ml worm cultures.

First day of culture:

1. *Prepare the media*: Pellet the bacteria in sterile 1 liter bottles by spinning at 4200 rpm for 15 min, resuspend the bacteria corresponding to each liter of bacterial culture in 500 ml S complete media and transfer to a sterile 2.8-liter flask in preparation for worm growth. Alternatively, pellets can be stored at

4°C for 1–2 weeks until use. To prevent contamination, set up the cultures using sterile technique in a tissue culture hood.

2. *Harvest the worms from the plates and seed the liquid culture*: Rinse the NGM/OP50 worm plates with sterile M9 and collect the worms in a 50-ml conical tube. Spin down the worms at 600 × *g* for 2 min in a clinical centrifuge to pellet. Remove the supernatant and resuspend the worms in 10 ml M9. Divide the worms between the two flasks containing OP50–1 in S complete media (from step 1). Shake the worms at 230 rpm in a cooling incubator maintained at 20°C for 3–4 days. Monitor the progress of the cultures carefully and harvest when the majority of worms are at the adult stage. Check the cultures by removing a 5-ml aliquot from the flask with a sterile pipette, spinning down the worms at 600 × *g* for 2 min, removing the supernatant, and washing the worms 1× with cold M9. You can then resuspend the worm pellet in a little amount of M9 and examine them on a dissecting scope after spotting a 4-µl aliquot onto a slide under a coverslip.

Harvesting the worms and isolating embryos:

1. Transfer each 500 ml culture of worms to a sterile 1 liter centrifuge bottle and spin for 7 min at 700 × *g*.

2. Aspirate off excess media and transfer worm slurry from each bottle to a 50-ml conical tube. Pellet at 600 × *g* for 3 min and aspirate off supernatant.

3. Combine worms into one 50-ml conical tube (not more than 10 ml of worms per conical tube) and wash by filling the conical tube with cold M9 buffer and pelleting again at 600 × *g* for 3 min.

4. Isolate the adults from the other larval stages and debris by performing a "sucrose float." Aspirate off the supernatant and resuspend the worm pellet in 25 ml of cold M9. Add 25 ml of cold 60% w/v sucrose dissolved in water. Mix by inverting and immediately spin at 1500 × *g* for 5 min. Initiate this spin right away or the worms will slowly desiccate in the sucrose.

5. During the spin, the adult worms will float to the top of the conical tube. Transfer the adults to a new conical tube with a transfer pipette and wash them once by filling the tube up with cold M9 and pelleting them at 600 × *g* for 3 min. Remove the supernatant and resuspend the worms in 25 ml of 0.1 M NaCl, mix by pipeting up and down and incubate on ice for exactly 5 min. After 5 min, aspirate off worms that have not settled (nongravid) in preparation for bleaching of gravid worms.

6. Aspirate off the supernatant and resuspend the worm pellet to 30 ml with 0.1 M NaCl. To this, add a mixture of 5 ml of 5 M NaOH and 10 ml of bleach [Fisher SS290–1 (4–6%), store sealed container, wrapped with parafilm at 4°C]. Vortex briefly at maximum speed and allow the mixture to stand at room temperature for 2 min.

7. Repeat vortexing every 2 min for a total of 8–15 min. Monitor the bleaching periodically by pipeting 4 µl samples onto a slide, covering with a coverslip and examining under a dissecting scope to assess degree to which worm debris is dissolved. Do not overbleach.

8. Centrifuge for 1 min at 800 × *g* at 4°C. Wash the pellet once by filling the conical tube up with ddH_2O (4°C) and repellet by spinning at 700 × *g* for 2 min.

9. Wash once more by filling up the conical tube with room temperature M9 and pelleting the embryos at 700 × *g* for 2 min.

10. Add 50 ml of M9 to the pellet and transfer the embryo pellet to a sterile 500 ml Erlenmeyer culture flask.

11. Shake in a temperature-controlled 22°C incubator until the embryos hatch (~12–24 h) yielding a synchronous culture of starved L1s.

b. Step 2: Synchronous Growth of DP38s in Liquid Culture

Two days prior to starting the synchronous culture: Seed 100 ml culture of OP-50–1 in LB plus 50 µg/ml streptomycin.

One day prior to starting the synchronous culture: Use the 100 ml culture to seed 6 × 1 liter overnight cultures of OP50–1 in LB plus 50 µg/ml streptomycin. This will be enough bacteria to start 6 × 500 ml worm cultures.

First day of synchronous culture:

1. *Prepare the media*: Pellet the bacteria from each 1 liter culture in a sterile 1 liter bottle by spinning the first liter at 4200 rpm for 15 min, adding the remaining 500 ml of culture to the bottle, and repeating the spin. Resuspend the bacteria from each bottle in 20 ml of S complete media and transfer to a sterile 2.8-liter flask containing 500 ml of S complete media. After this step, you should have six flasks containing S complete media plus the bacteria from 1 liter of culture.

2. *Seed the synchronous culture*: Pellet the L1s from step 11 above by spinning at 600 × *g* for 3 min. Bring up to 50 ml with sterile M9 and repellet.

3. Resuspend the worms in 12 ml of sterile M9.

4. Seed each flask with the equivalent of 50–100 µl of pure L1 pellet.

5. Shake the worms at 230 rpm in a cooling incubator maintained at 20°C for 3–4 days. Monitor the progress of the cultures carefully by periodically removing a 5-ml aliquot from the flask with a sterile pipette, spinning down the worms at 600 × *g* for 2 min, removing the supernatant, and washing the worms 1× with cold M9. Examine the resuspended worm pellet after spotting a 4-µl aliquot onto a slide under a coverslip. Harvest four of the flasks for bombardments (see below) when the worms are at the L4 stage (do not use young adult worms). Each flask should yield enough worms for 8–10 heptad bombardments (depending on worm density).

6. If you want to maintain the liquid culture, continue to incubate the remaining two flasks until the worms are adults; harvest embryos by bleaching (as described above) to seed the next round of synchronous culture.

c. Step 3: Modifications for Large-Scale Bombardment Using the Hepta-Adaptor

When bombarding worms cultured in liquid, a *hepta-adaptor (Bio-Rad #165–2225)* can be attached to the bombardment apparatus allowing greater quantities of worms to be bombarded at one time.

1. Pellet the L4 culture (as prepared above) at 700 × *g* for 5 min. Worms should be washed with M9 to remove excess bacteria. After spinning 600 × *g* for 2 min, remove as much supernatant as possible in preparation for distribution of worms onto the bombardment plates.
2. On each bombardment plate (10 cm), mark the approximate position of each macrocarrier in the hepta-adaptor using a pen.
3. Distribute 100 µl clumps of washed L4 worms onto these marked areas.
4. Prepare DNA beads and macrocarriers as described in the small-scale protocol, but use 10 µg of DNA for each heptad bombardment. Also, macrocarrier disks should not be placed in individual metal holders (as described in the small-scale protocol); instead, they are inserted directly into the hepta-adaptor unit.
5. Position each bombardment plate on the shelf directly below the hepta-adaptor (~2 cm). Line up each worm clump with each macrocarrier in the hepta-adaptor apparatus. Verify that the air outlets are also in alignment.
6. Bombard using 1350 psi rupture disks and hepta-adaptor stopping screens (Bio-Rad 1652226). Stopping screens can be used for several bombardments with the same construct. Because of the hepta-adaptor setup, the rupture disks will break closer to 2000 psi.
7. Let worms recover for 1–2 h at 20°C.
8. Distribute worms from one heptad bombardment plate onto 10 small (60 mm) plates and incubate at 20°C for 2–3 weeks. Expect approximately five moving lines per heptad bombardment.

3. Current Challenges: Endogenous Gene Replacement

In other systems, such as yeast and mice, gene targeting with homologous recombination can replace endogenous genes with constructed transgenes or knock them out to create null mutations. While homologous recombination occurs with low incidence in *C. elegans* following large-scale bombardment (Berezikov *et al.*, 2004) or injection of DNA into oocyte nuclei (Broverman *et al.*, 1993), its low frequency has prevented this method from being extensively utilized. By scaling up biolistic bombardment methods, Berezikov and colleagues have demonstrated, for two independent loci, that ~300 transformants are required to isolate one

homologous recombination event. Future work improving the frequency of homologous transformation events either by technological advances or by identification of mutants that facilitate efficient recombination will be an important advance.

4. Controlling Growth Temperature to Influence Expression

Germline silencing is temperature sensitive and weakest at 25°C, which is effectively the highest temperature at which worms will remain fertile (Strome *et al.*, 2001). For difficult-to-obtain transgenic lines, growth at 24.5°C can be used to maximize the chances of obtaining a line that expresses the transgene. However, in most cases, returning these transgenic lines to lower temperatures results in rapid silencing that can sometimes be permanent. The use of these strains has many limitations. For example, such transgenes cannot be used in temperature-sensitive mutants due to silencing at the permissive temperature. In general, we recommend that transgenic strains initially be isolated at 20°C. In cases where fluorescence is absent, strains should be shifted to 24.5°C and rescreened shortly thereafter.

B. Making Dual/Triple Fluorescent Marker Lines by Mating

It is usually straightforward to generate multimarker lines once single fluorescent transgenic lines have been established. To do this, males generated for one of the fluorescent strains are crossed to hermaphrodites homozygous for the second fluorescent marker (see Fig. 6). The F2 progeny from this mating are singled and their progeny are screened to identify lines homozygous for both fluorescent markers. In our experience, GFP and mCherry provide an optimal combination of fluorescent markers.

1. Generating and Maintaining Males

Male worms, which have a single X chromosome (XO), arise at low frequency in a hermaphrodite (XX) population by chromosome nondisjunction. Below we describe our version of a commonly used heat shock protocol to increase the frequency of males in strains bearing fluorescent transgenes.

1. Set up an incubator (or water bath) at 30°C and another at 31.5°C.
2. Transfer 10 L4 hermaphrodites onto each of two 10-cm plates seeded with OP50 bacteria.
3. Parafilm and place one plate at 30°C and one at 31.5°C for 8 h.
4. Remove excess condensation and return plates to 20°C incubator for 3–4 days.

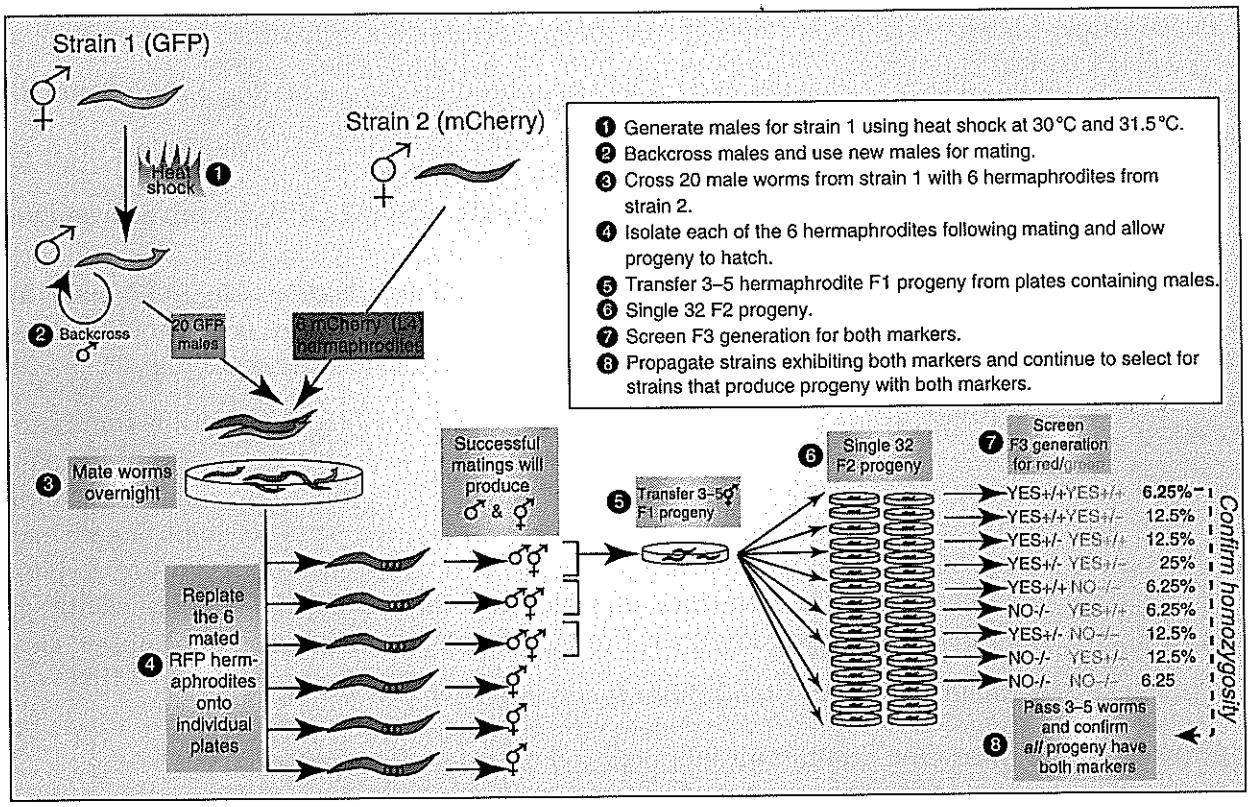


Fig. 6 Standard mating protocol to generate multimarker lines. Begins by generating males from the first strain by subjecting L4 hermaphrodites to heat shock at 30 and 31.5° C. Allow hatching of progeny and isolate males for backcrossing. Backcross males and use newly isolated males for mating. Cross 20 male worms (strain 1) with 6 hermaphrodite worms (strain 2). Single the six hermaphrodites onto individual plates and allow progeny to hatch. Transfer three to five hermaphrodite F1 progeny from plates that have ~50% males (indicative of successful mating). Single out 32 F2 progeny and screen their progeny (the F3 generation) for both markers to determine the genotype of the F2 parent. The expected incidence for each genotype is listed (assuming the markers are on different chromosomes). Propagate strains where all F3 progeny express both markers, indicating that the F2 parent was homozygous for both markers. If the F2 parent was heterozygous for a marker, then ~1/4 of its progeny will lack the marker. Check again at the next generation to confirm that both markers are homozygous. (See Plate no. 26 in the Color Plate Section.)

5. Check plates for males when the progeny are L4 stage. Male worms are easily distinguished by their smaller size and the presence of a copulatory apparatus or "hook" at the end of the tail.

This method generally only produces a few males per plate, and due to heat shock these males may not mate efficiently. Therefore, it is best to backcross the males before using them for mating. Male sperm out compete hermaphrodite sperm (LaMunyon and Ward, 1995; Ward and Carrel, 1979). Since 50% of male sperm carry the X chromosomes and 50% do not, successfully mated hermaphrodites produce ~50% male progeny.

2. Backcrossing Males

1. Place 5–20 males and 6 L4 hermaphrodites onto a mating plate [60 mm dish seeded with small spot (10 µl of saturated OP50 culture)].
2. Incubate at 16 or 20°C overnight.
3. Move hermaphrodite worms to a standard NGM/OP50 60-mm dish and look for male progeny after several days.
4. Male worms can be maintained by setting up a mating with 20 L4 males and 6 L4 hermaphrodites on a standard 60-mm dish seeded with bacteria at each generation.

If only a few males are generated by the heat shock, you can maximize your chance of "capturing" the males by mating them with an excess (~20) of hermaphrodites for 24 h and then plating the hermaphrodites, three onto a plate, to simplify identification of plates with male progeny. It is often worth trying to make males from both of the strains containing the two transgenes that you are trying to combine, since the frequency of males and their ability to mate efficiently can vary between strains.

3. Crossing Males from One Strain with Hermaphrodites from a Strain Carrying a Second Fluorescent Marker

1. Once you have males from one strain, cross the two strains by placing 10 L4 males from the first strain and 6 L4 hermaphrodites from the second strain on a mating plate.

2. Allow them to mate for 24–36 h at 20°C. If one strain requires growth at 16°C, allow them to mate longer at 16°C.

3. Single the hermaphrodites to fresh plates (six separate plates) and allow progeny to hatch. A plate carrying ~50% males indicates successful mating. Throw away the other plates.

4. The hermaphrodites on the plates with ~50% males are cross progeny and should be heterozygous for both fluorescent markers. Make two small plates with 3–5 heterozygous worms each and let these lay progeny to obtain the F2 generation.

5. The F2 worms will have a variety of genotypes and can be heterozygous, homozygous, or null for each fluorescent marker. If the two markers are on different chromosomes, 1/16 of the F2 progeny will have neither marker, 3/8 will be null for one marker and either heterozygous or homozygous for the second marker, 1/4 will be heterozygous for both markers, 1/4 will be homozygous for one marker and heterozygous for the second marker, and 1/16 will be homozygous for both markers. To identify the desired double homozygotes, we single the F2

worms onto individual plates and use fluorescence microscopy to screen expression in the gonad/embryos of their adult progeny (screening procedure described below). The progeny of the worms homozygous for both markers (~1/16 of the plates) will all have both markers. Depending on how many F2 worms you single, you may not find a plate where this is the case. The next best thing is plates where the parental F2 was homozygous for one marker and heterozygous for the second marker. The progeny of these worms will all have the first marker and ~1/4 of the progeny will lack the second marker. If you do not get a homozygote the first time, you can single worms from one of these plates and screen again in the next generation to homozygose the second marker—this time 1/4 of the worms should be homozygous for both markers. If the two markers are on the same chromosome, obtaining a double homozygote requires a recombination event. Recombinants can be straightforward to obtain if the markers are far apart—or nearly impossible to obtain if they are closely linked.

4. Screening for Strains Homozygous for Two Fluorescent Markers

1. Single ~32 F2 L4 hermaphrodites onto individual plates and incubate until there are adult progeny on the plate.

2. Whole-mount adult hermaphrodites from each plate. To whole mount, spot 2 µl of M9 onto a glass slide, transfer worm into M9 spot, place coverslip gently on top, and lightly press down on the edges of the coverslip to restrain worm movement (careful not to squish worm). Place it under the microscope and screen for the presence of both fluorescent markers in the germline/embryos. If one or both markers are absent from any of the progeny, throw the plate away. If both markers are present in all progeny (say >32 progeny), then the strain is most likely homozygous for both markers. In this case, pass three to five worms onto a new plate and screen again at the next generation to confirm that the line is homozygous for both markers. If one marker is present in all progeny but the second is absent in ~1/4 of the progeny, keep the plate but note that the parent was likely heterozygous for the second marker. Single worms from this plate and check again for double homozygotes in the next generation.

C. Benefits and Challenges with Multimarker Lines

Establishing dual color *C. elegans* lines typically results in beautiful images and valuable data. However, the usefulness of double or triple *single*-color lines is often overlooked. When two fluorescently marked proteins are spatially distinct in distribution and similar enough in signal intensity that a single acquisition setting can be employed to image both; dual single-color strains can be incredibly useful.

A nice example of this is a line that expresses GFP-γ-tubulin and GFP-histone, marking the spindle poles and chromosomes, respectively (see Fig. 7). This line offers the benefit of requiring less light exposure, compared to a dual colored line marking the same cellular components, which allows for longer filming. For laboratories with limited access to elaborate microscope facilities, these types of lines permit sophisticated analysis of multiple proteins of interest at the same time, using a basic fluorescence microscope setup. In addition, these lines can be crossed with other fluorescent lines to allow visualization of three or four cellular components simultaneously.

V. Using Fluorescent Worm Strains

A. Confirming Functionality of Transgenes

Before using fluorescent fusion proteins to obtain quantitative data, it is important to confirm that the fusion is functional and therefore likely to reflect the behavior of the endogenous protein. If there is an appropriate mutant available,

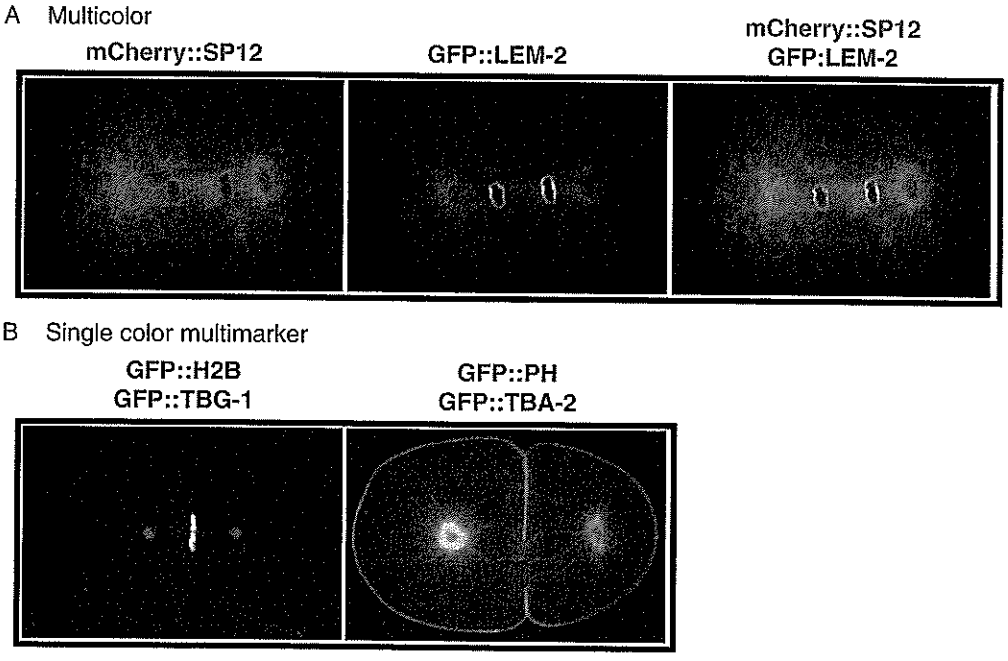


Fig. 7 Multicolor versus single-color multimarker strains. (A) In this strain, two proteins with a partially overlapping distribution are labeled with different fluorophores. mCherry::SP12 (left panel) labels the ER lumen. GFP:LEM-2 also localizes to the ER, but unlike SP12, is specifically enriched in the inner nuclear membrane (A. Audhya, Oegema Laboratory, unpublished strain). (B) Two examples are presented of single-color, multimarker strains to illustrate their usefulness when the spatial distributions of the two marked populations are distinct. The left panel shows GFP marking in both chromosomes (GFP::H2B) and centrosomes (GFP::TBG-1) at metaphase [strain TH32 (Cheeseman *et al.*, 2004)]. The right panel shows the plasma membrane (GFP::$PH^{PLC\delta1}$) and microtubules (GFP::TBA-2) in an embryo undergoing cytokinesis [strain OD73 (Audhya *et al.*, 2005)]. (See Plate no. 27 in the Color Plate Section.)

introduction of the GFP fusion into the mutant background can accomplish this goal. For transgenes expressed using the *pie-1* promoter, maternal effect or conditional mutants are ideal, since worms homozygous for a null mutation in an essential gene will often not survive to the developmental stage when the *pie-1* regulatory sequences drive expression. Below we discuss the use of RNAi-resistant (RR) transgenes, a useful alternative approach when an appropriate mutant is not available.

1. RR Forms of Transgenes

The generation of RR transgenes can be useful to test the localization and function of an introduced fusion protein in the absence of the endogenous protein (Fig. 8). RR transgenes are engineered to contain silent mutations that diverge from the original gene at the nucleotide level but not at the protein level. The first step in generating an RR transgene is to identify a small region of the endogenous gene against which an effective dsRNA can be made. We typically choose a region that is 300–500 bp in size, flanked by convenient restriction sites, and composed of mostly exonic (coding) sequence. Before recoding, it is important to make a dsRNA against this region and ensure that injection of this RNA effectively depletes the endogenous protein. In some cases, use of an RNA against the 3′ UTR of the endogenous gene (not present in transgenes driven by the *pie-1* regulatory sequences) may suffice, obviating the need to make a special transgene. However, in many cases, the 3′ UTRs are too short to be effective. In general, longer dsRNAs improve depletion efficiency (although they make reencoding more expensive).

After an appropriate target region is selected, the nucleotide sequence must be "recoded" without altering the original amino acid sequence or overall codon bias. To achieve this, the simplest method is to shuffle alternative codons encoding the same amino acid within the region. For example, if the first serine in the selected region is encoded by AGU and the second serine in the region is encoded by AGC, swap the two codons. Continue swapping codons until the coding sequence for all the serine residues have been altered. This approach should be repeated for each amino acid within the targeted sequence until there are no long (>10 bp) stretches of homology between original and reencoded sequence at the nucleotide level. The final sequence can then be compared with the endogenous sequence using a web-based program such as Graphical Codon Usage Analyser (http://gcua.schoedl.de/) to verify that (1) the amino-acid sequence remains unchanged, (2) there are no stretches of conserved nucleotide sequence >10 bp, and (3) the overall codon bias remains the same. Any introns present in the targeted region should be replaced entirely with synthetic introns (see Section II.B.2, for example, synthetic intron sequences). The recoded region is then synthesized (usually by a commercial vendor) and introduced into the transgene in place of the original coding sequence via the flanking restriction sites.

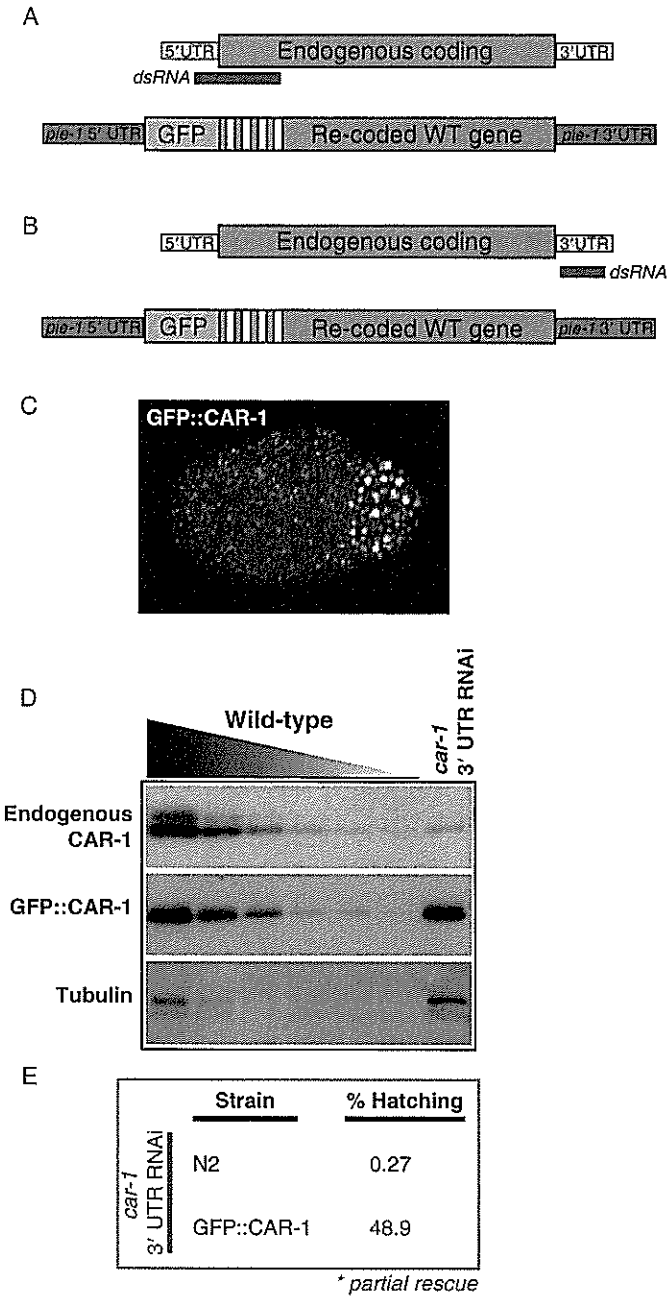


Fig. 8 Two RNAi strategies to selectively deplete the endogenous protein without affecting expressed tagged fusions. (A) The nucleotide sequence of part of the coding region in the introduced transgene is modified so that a dsRNA directed against part of the endogenous coding region can be used to selectively deplete the endogenous protein. (B) A dsRNA directed against the 3′ UTR of the endogenous gene selectively targets the endogenous mRNA because the transgene utilizes the *pie-1* 3′ UTR. (C–E) Using 3′ UTR RNAi to test functionality of GFP fusions. Panels C–E have been adapted from Audhya *et al.* (2005). (C) GFP::CAR-1, like endogenous CAR-1, localizes to cytoplasmic particles and P-granules. (D) Western blots of extracts from GFP::CAR-1 expressing worms injected with 3′ *car-1* UTR dsRNA, probed using anti-CAR-1 and anti-GFP antibodies reveal the selective depletion of the endogenous protein without alteration of the levels of the GFP fusion. Serial twofold dilutions of extracts prepared from untreated worms were loaded to quantify depletion. (E) Expression of GFP::CAR-1 rescued embryonic viability following selective depletion of endogenous CAR-1. (See Plate no. 28 in the Color Plate Section.)

Once an RR transgene has been generated and a strain produced by integration, it is possible to use the RNA against the recoded region to selectively deplete the endogenous protein without altering levels of the transgenic protein, allowing the functionality of the transgenic protein to be tested. Once it is established that a fluorescent fusion with a coding region rescues function, it is also possible to compare the functionality of the control RR transgene with that of transgenes containing specific mutations of interest. A nice example of how this technique has been employed is presented in Ozlu *et al.* (2005) examining the function of TPXL-1 in targeting of AIR-1 to the mitotic spindle.

B. Available Worm Strains for Imaging in the Gonad and Early Embryo

A number of useful worm strains, carrying fluorescent transgenes, have been published and are available for general use. These strains include markers for studying various cellular components, including centrosomes, microtubules, kinetochores, chromosomes, actin cytoskeleton, microtubule-associated proteins, polarity determinants, and membrane-bound organelles. To view a comprehensive list of currently available strains (see Fig. 9).

C. Practical Techniques for Gonad/Embryo Imaging: Specimen Mounting and Drug Treatments

Mounting techniques for imaging the first embryonic division include *ex vivo* methods, either on agarose pads or in meiosis media, or *in utero* methods, via a worm whole mount. Each technique will be discussed briefly below.

1. Mounting Dissected Embryos for Imaging under Compression

Below we outline the standard method used to image living embryos. In this method, the embryos are dissected out of the mother and are mounted under compression on an agarose pad.

1. Make an agarose pad for mounting embryos by placing a slide between two slides that are each covered with two strips of laboratory tape, so that their tops are slightly higher than the slide in the middle. Put an M&M-sized drop of melted agarose (melted by placing an eppendorf tube containing 2% agarose in a heating block at 95°C) on the center of the middle slide. After waiting for a second or two for the agarose to slightly cool, place a second slide crosswise over the agarose drop, so that it rests on the tape-covered slides. This should generate a circular pad of agarose on the center of the slide. After the agarose has cooled, pick up the agarose pad-slide sandwich and twist the two slides until you have one slide with a thin pad of agarose on it ready for embryo mounting. *Note:* The agarose breaks down over time at high temperatures reducing its usefulness for making pads, so put a new aliquot into the heating block every hour or so.

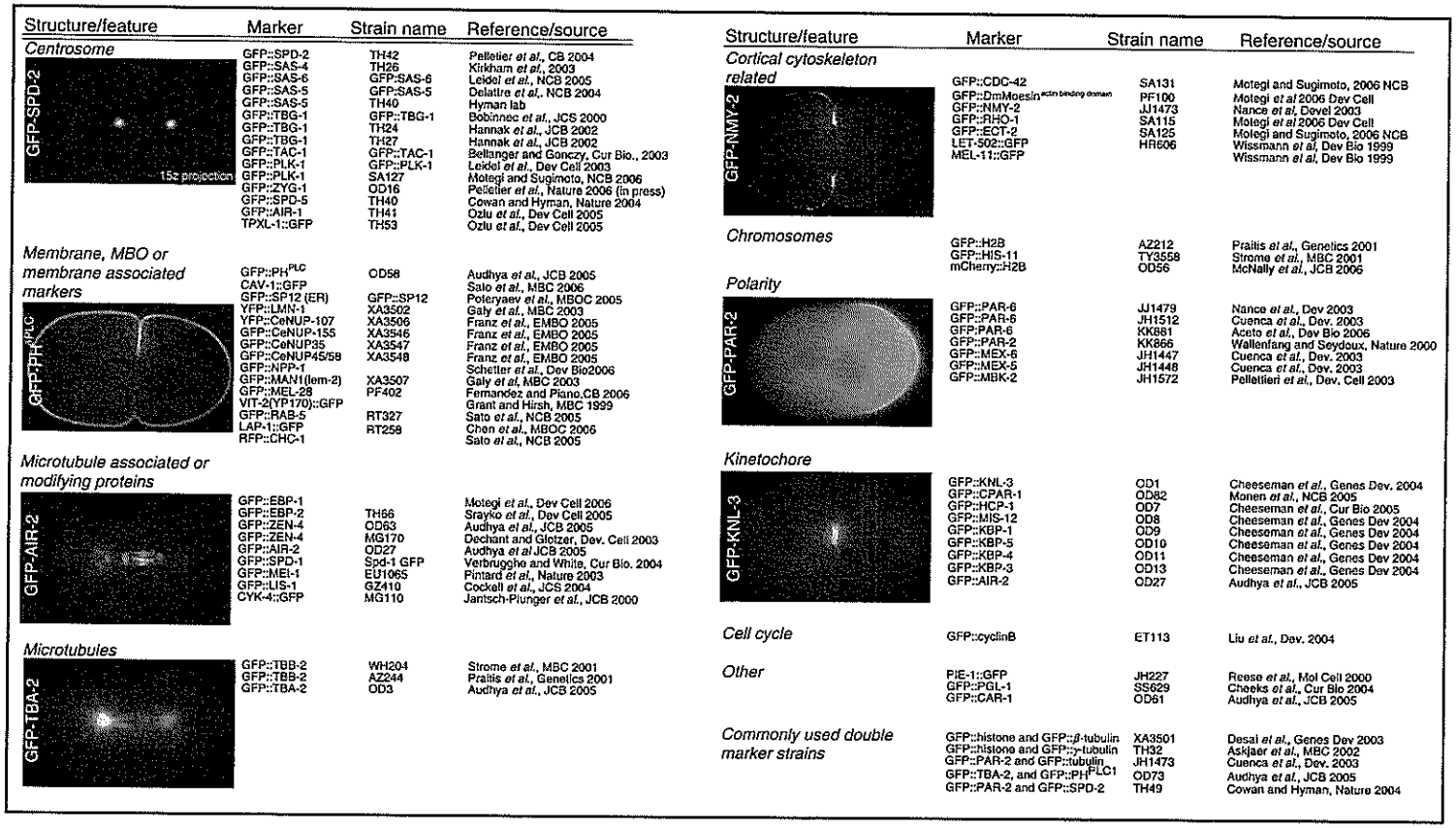


Fig. 9 Published fluorescence strains for imaging in the gonad and early embryo. The fluorescence marker::gene, strain name (when available), and reference/source are listed for all currently *published* germline expression strains. Fluorescent strains are grouped according to the cellular features that they mark; these include centrosomes, membranes/membrane-bound organelles, microtubules, microtubule-associated proteins, cytoskeleton, chromosomes, polarity determinants, kinetochores, cell cycle regulators, and miscellaneous targets.

2. To obtain embryos, transfer an adult worm to a specimen watch glass (Electron Microscopy Sciences #71570–01) containing a small amount (~1–2 ml) of M9. Hold the worm at one end with fine forceps (Dumont-Dumostar #10570) and use a scalpel to cut the worm in half. Grab each half of the worm with the forceps and scrape the embryos out of the worm with the scalpel, like toothpaste from a tube. Using a scalpel with a small blade (Becton-Dickinson #371615) can be very helpful for this.

3. Pick up the embryos with a mouth pipette and transfer them to the agarose pad, trying to minimize the amount of liquid transferred. Mouth pipettes, with a diameter slightly larger than an embryo, are pulled from 25 µl capillaries over a Bunsen burner. The mouth pipette/capillary holders can be found in the capillary packages.

4. After transferring the embryos to the pad, use the mouth pipette to remove some of the excess liquid if necessary and then use an eyelash tool (an eyelash affixed to the end of a toothpick with glue or nail polish) to herd the desired embryos together (making them easier to find under the microscope).

5. Place an 18 mm × 18 mm coverslip over the embryos and transfer the slide to the microscope for imaging.

An alternative method that can be simpler for beginners is to place a worm in a small drop (2–3 µl of M9) on an 18 mm × 18 mm coverslip and use a pair of fine needles to dissect the embryos from the worm on the coverslip. The coverslip can then be inverted onto the agarose pad and the embryos imaged.

2. Imaging the Gonad in Anesthetized Worms

Below we describe our standard protocol for imaging the gonad in anesthetized worms. In some cases, the protocol can also be used to image the division of embryos "*in utero,*" which can be useful if the embryos are osmotically sensitive and lyse when imaged using the standard procedure described above.

To anesthetize the worms for *in utero* imaging:

1. Make up a fresh mixture of 1 mg/ml Tricaine (ethyl 3-aminobenzoate methanesulfonate salt) and 0.1 mg/ml of tetramisole hydrochloride in M9.

2. Place worms into a pool of anesthetic (a depression slide works well for this) for 15–30 min, or until worms stop moving.

3. Transfer the anesthetized worms to an agarose pad (prepared as described above) and carefully place a coverslip on top. The orientation of the anesthetized worm on the agarose pad is somewhat random, obscuring a clear view of the embryos in some cases. Therefore, it is recommended that several worms be anesthetized for each condition.

3. Mounting for Meiosis Imaging

Meiotic embryos do not tolerate compression and are osmotically sensitive, since the eggshell is not yet fully formed. Nevertheless, it can be convenient to image them *ex vivo* to obtain a high-resolution view of meiotic events, such as meiotic chromosome segregation, in the embryo. To do this, we use the alternative mounting technique described below.

1. First, assemble a meiosis filming chamber as follows:
   (a). Use two strips of double-sided tape to secure a 60 mm × 20 mm coverslip onto the top of an aluminum "slide" with a hole in the center. The aluminum slide is a holder that provides structural rigidity to the coverslip, while allowing imaging through the hole.
   (b). Apply a ring of vaseline on top of the coverslip, inside the area defined by the hole in the aluminum slide.
   (c). Pipet 8 μl of meiosis media into the center of the vaseline ring.
2. Place three adult worms into the meiosis media drop and dissect out the embryos.
3. Push worm debris to the edges of the drop, leaving the embryos centered.
4. Place an 18 mm × 18 mm coverslip on top of the drop. The vaseline should prevent the embryos from experiencing compression.
5. Place the slide on the microscope stage and image with the lens closest to the large coverslip (through the hole in the aluminum slide).

4. Drug Treatments

The eggshell of the early embryo serves as a barrier, making introduction of experimental drugs, such as nocodazole, difficult at this stage. While several laboratories have had some success introducing drugs into embryos by cracking the eggshell or incubating dissected embryos (with intact eggshells) in drugs (Encalada *et al.*, 2005; Kurz *et al.*, 2002; Stear and Roth, 2004; Strome and Wood, 1983), the reproducibility of these approaches can be variable. We have had success introducing both nocodazole and latrunculin A into early embryos by dissecting worms in meiosis media, supplemented with drug. Because there is a delay between fertilization and eggshell formation, newly fertilized embryos can still take up the drug until approximately the end of meiosis II. These embryos can be filmed under meiosis imaging conditions or on an agarose pad following drug exposure.

For introduction of drugs at later timepoints, we have had some success with a chitinase treatment protocol that maintains the eggshell in a permeable state. In this protocol, three worms are dissected in 8 μl egg salts (48 mM NaCl, 118 mM KCl) on a 24 mm × 60 mm coverslip on which a drop of 4 μl polylysine (1 mg/ml) has been dried in an oven for 10 min. The polylysine spot helps to immobilize the embryos through subsequent treatments. The coverslip is mounted

with a ring of vaseline on an aluminum slide, as described above for meiosis filming. The buffer is removed with a mouth pipette and replaced with 8 µl of 9:1 ddH_2O:bleach by volume. After 2 min, the bleach solution is replaced with 8 µl egg salts buffer, followed sequentially by 8 µl L-15 blastomere culture medium (Edgar, 1995) and 8 µl chitinase (5 U/ml in L-15 blastomere culture medium). After 4 min, the chitinase solution is replaced with 8 µl L-15 blastomere culture medium. Drugs can be introduced by replacing this medium with 8 µl L-15 blastomere culture medium containing the drug.

D. Guidelines for Live Imaging of *C. elegans* Embryos

Once the specimens are prepared, the *C. elegans* gonad and early embryo are imaged in a fashion similar to other living specimens, and are subject to the same considerations. We remind experimenters to choose conditions to favor the health and viability of the embryo (temperature, illumination intensity) and to optimize imaging parameters (optics, filters, on-chip binning). Because general recommendations for live imaging are well described elsewhere, we will limit our discussion to *C. elegans*-specific considerations for live imaging.

The *C. elegans* gonad and early embryos have been imaged with great success using both wide-field and confocal microscopes. Ideally, the choice of imaging system should be tailored for each experiment, depending on the goal of the study. When imaging the cytoskeleton or the cell cortex, we recommend using a spinning disk confocal microscope. The spinning disk maximizes the number of confocal images that can be acquired before bleaching or phototoxicity become problematic (Audhya *et al.*, 2005; Maddox *et al.*, 2003). For chromosome or centrosome imaging, a standard wide-field microscope or a spinning disk confocal can be used to acquire a short *z*-series (5–6 planes, 2 µm intervals) through the middle region of the embryo. For fixed embryos analysis, deconvolution wide-field imaging at full resolution is the method of choice (Agard, 1984; Cheeseman *et al.*, 2004; Maddox *et al.*, 2005; Monen *et al.*, 2005). Other methods of imaging, such as multiphoton analysis, have also been successfully used to image *C. elegans* embryos (Strome *et al.*, 2001).

Filters used for fluorescence imaging should be closely matched to the peak wavelengths of the fluorophores being used to avoid the need for longer exposure conditions, which may be harmful to the specimen. For GFP-only imaging, we use a 488-nm band-pass filter and for dual GFP and mCherry imaging, we use 488/568 nm dual band-pass dichroic with individual band-pass excitation and emission filters mounted in filter wheels. For simultaneous imaging of two fluorescent proteins, we prefer GFP/mCherry to CFP/YFP since the excitation light for CFP is not well tolerated by *C. elegans* embryos.

For high-resolution live imaging of *C. elegans* embryos, 100× oil immersion (1.4 NA), 60× oil immersion (1.4 NA), and 60× water immersion (1.2 NA) objectives are most commonly used. Water immersion lenses can reduce spherical aberration, which becomes noticeable when imaging deep in ~20 µm thick embryos.

For maximal detail, the net magnification must be sufficient to capture full resolution onto the detector, which is typically a charge coupled detector camera. Magnification beyond this optimum reduces intensity without providing any added information. Typically, the theoretical resolution limit (~0.25 μm for green light) is split over three pixels on the camera (i.e., for a camera with 6.45 μm square pixels, 77× magnification is optimal). The 1.5× auxiliary magnification, built into the microscope (often referred to as an optivar), can be used to increase net magnification onto the detector by an additional 50%.

In practice, we rarely image living embryos at full resolution because many GFP transgenes are expressed at low levels and the signal is limiting. Instead, we sacrifice spatial resolution by using 2 × 2 on-chip binning to amplify the signal without increasing noise. Binning converts four adjacent pixels into a single large pixel (for a 6.45-μm square camera, after binning the total pixel number will be reduced fourfold and each pixel will be 12.9 μm^2); effectively, binning results in a fourfold increase in signal-to-noise and a twofold decrease in spatial resolution. Binning is essential for many of our imaging procedures because it allows the use of illumination and exposure conditions that permit long-term imaging of embryos without significant photodamage.

VI. Summary

The *C. elegans* germline has recently emerged as a powerful model system for studying meiosis and mitosis. The advantages of this system include reproducible RNAi-mediated protein depletion, stereotypical gonad morphology, and the rapid and reproducible progression of the first embryonic cell divisions. The advent of fluorescent proteins engineered for *C. elegans* expression and the means to avert the germline silencing of transgenes has made it possible to image fluorescent proteins in this system. In this chapter, we have outlined methods currently used to engineer fluorescent transgenes for germline expression, bombardment methods for the generation of stable lines, approaches to verify transgene function and expression, methods to generate multimarker lines, techniques for mounting worms and embryos, and basic imaging guidelines. In addition, we provide a list of currently available vectors and strains for live imaging analysis in the gonad and the early embryo.

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Appendix

Media and Supplements

1. *LB (2×)*

   100 g of LB
   1800 ml ddH_2O
   *Autoclave 35 min

2. *LB plates (1 liter)*

   500 ml ddH_2O
   15 g agar
   Add 450 ml 2× LB to the bottle
   *Autoclave 35 min

3. *Meiosis media*

   Stocks needed to make media:
   500 mg/100 ml inulin in culture safe H_2O (autoclaved)
   0.25 M HEPES pH 7.4 (5.95 g/100 ml)
   Leibowitz L-15 media
   Heat-inactivated FBS

   For 10 ml of meiosis media:
   6 ml of L-15 media
   1 ml of HEPES (0.25 M pH 7.4)
   1 ml inulin solution
   2 ml FBS

4. *M9 (2 liter)*

   10 g NaCl
   12 g Na_2HPO_4
   6 g KH_2PO_4
   0.5 g $MgSO_4 \cdot 7H_2O$
   Add water to 2 liter
   *Autoclave 35 min

5. *NGM agarose plates (1 liter)*

3 g NaCl
25 g agarose
2.5 g peptone
1.0 ml cholesterol (5 mg/ml in EtOH)
975 ml ddH_2O
*Autoclave 35 min and place in 55°C water bath
*When cooled sterilely add:
1.0 ml 1 M $CaCl_2$
1.0 ml 1 M $MgSO_4$
25 ml 1 M KH_2PO_4 (pH 6.0)

6. *Nystatin solution (500 ml)*

5 g nystatin
250 ml EtOH
250 ml 7.5 M NH_4Ac
*Filter sterilize and store at −20°C

7. *Peptone plates (1 liter)*

1.2 g NaCl
20 g bactopeptone
25 g agar
*Autoclave then sterilely add:
1 ml 5 mg/ml cholesterol
1 ml 1 M $MgSO_4$
25 ml 1 M KH_2PO_4 pH 6.0

8. *Potassium citrate (1 M), pH 6.0*

268.8 g tripotassium citrate
26.3 g citric acid monohydrate
Add water to 900 ml
*Adjust pH to 6.0 using 10N KOH and bring up to 1 liter
*Autoclave and store at room temperature

9. *S basal (per liter)*

5.9 g NaCl
50 ml of 1 M KH_2PO_4 pH 6.0
1 ml of cholesterol (5 mg/ml in EtOH)
*Autoclave and store at room temperature

10. *S basal (complete media):*

To S basal 500 ml bottle add:
5 ml 1 M potassium citrate pH 6.0
5 ml trace metals solution
1.5 ml 1 M $MgSO_4$
1.5 ml 1 M $CaCl_2$

11. *Trace metals solution:*

Disodium EDTA	1.86 g	(5 mM)
$FeSO_4 \cdot 7H_2O$	0.69 g	(2.5 mM)
$MnCl_2 \cdot 4H_2O$	0.20 g	(1 mM)
$ZnSO_4 \cdot 7H_2O$	0.29 g	(1 mM)
$CuSO_4 \cdot 5H_2O$	0.025 g	(0.1 mM)

*Dissolve in 1 liter water; aliquot into 50 ml conical tubes and store in dark

Bombardment reagents

1. Bio-Rad 1.0 μm gold beads (Cat# 1652263)
2. Stopping screens Cat#1652336
3. Macrocarriers Cat# 9202964 120502
4. Rupture discs Cat# 1652330
5. 50% glycerol (filter sterilized)
6. pAZ-based plasmid for transforming (uncut) ~1 mg/ml (1 μl per bombardment)
7. 2.5 M $CaCl_2$ (sterile filtered)
8. 1.0 M spermidine (Sigma S-0266 in water, stored at −20°C)
9. 70% EtOH
10. 70% isopropanol
11. 100% EtOH
12. Sterile water

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