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METHODS IN MOLECULAR BIOLOGY™

John M. Walker, SERIES EDITOR

METHODS IN MOLECULAR BIOLOGY™

C. elegans

Methods and Applications

Edited by

Kevin Strange

*Departments of Anesthesiology, Molecular Physiology
and Biophysics, and Pharmacology
Vanderbilt University Medical Center
Nashville, TN*

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Preface

Molecular biology has driven a powerful reductionist, or "molecule-centric," approach to biological research in the last half of the 20th century. Reductionism is the attempt to explain complex phenomena by defining the functional properties of the individual components of the system. Bloom *(1)* has referred to the post-genome sequencing era as the end of "naïve reductionism." Reductionist methods will continue to be an essential element of all biological research efforts, but "naïve reductionism," the belief that reductionism alone can lead to a complete understanding of living organisms, is not tenable. Organisms are clearly much more than the sum of their parts, and the behavior of complex physiological processes cannot be understood simply by knowing how the parts work in isolation.

Systems biology has emerged in the wake of genome sequencing as the successor to reductionism *(2–5)*. The "systems" of systems biology are defined over a wide span of complexity ranging from two macromolecules that interact to carry out a specific task to whole organisms. Systems biology is integrative and seeks to understand and predict the behavior or "emergent" properties of complex, multicomponent biological processes. A systems-level characterization of a biological process addresses the following three main questions: (1) What are the parts of the system (i.e., the genes and the proteins they encode)? (2) How do the parts work? (3) How do the parts work together to accomplish a task?

Nonmammalian model organisms such as *Eschericia coli*, *Saccharomyces*, *Caenorhabditis elegans*, *Drosophila*, *Danio rerio*, and the plant *Arabidopsis* have become cornerstones of systems biology research. They have been likened to the Rosetta stone *(4)*, which provided modern scholars with the tools needed to decipher Egyptian hieroglyphics. Similarly, model organisms provide investigators the experimental tools necessary to decipher the genetic code that underlies complex physiological processes common to all life.

C. elegans provides a particularly striking example of the experimental utility of nonmammalian model organisms. Worms have a short life cycle (2–3 d at 25ºC), produce large numbers of offspring by sexual reproduction, and can be cultured easily and inexpensively in the laboratory. Sexual reproduction occurs by self-fertilization in hermaphrodites or by mating with males. Self-fertilization allows homozygous animals to breed true and greatly facilitates the isolation and maintenance of mutant strains, whereas mating with males allows mutations to be moved between strains. The reproductive and laboratory culture characteristics

of *C. elegans* make it an exceptionally powerful model system for forward genetic analysis. Mutagenesis and genetic screening allow unbiased identification of genes underlying a biological process of interest, allow the genes to be ordered into pathways, and can provide important and novel mechanistic insights into the molecular structure and function of proteins.

In addition to forward genetic tractability, *C. elegans* also has a fully sequenced and well-annotated genome. Genomic sequence and virtually all other biological data on this organism are assembled in readily accessible public databases (e.g., WormBase; http://www.wormbase.org). Numerous reagents, including mutant worm strains and cosmid and YAC clones spanning the genome, are freely available through public resources. Creation of transgenic worms is relatively easy, inexpensive, and rapid requiring little more than injection of transgenes into the animal's gonad or bombardment with DNA-coated microparticles. *C. elegans* gene expression can be specifically and potently targeted for knockdown using RNA interference, either at the single worm level by injection of double-stranded RNA, or at the population level by feeding worms double-stranded RNA-producing bacteria. Finally, *C. elegans* is a highly differentiated animal but is comprised of less than 1000 somatic cells. This relatively simple anatomy greatly facilitates the study of biological processes and has made it possible to trace the lineage of every adult cell beginning with the first cell division *(6,7)*, and to generate a complete wiring diagram of the 302 neuron adult hermaphrodite nervous system *(8)*.

A wealth of methodology for the study of *C. elegans* is described online and in the printed literature. The goal of *C. elegans: Methods and Applications* is to provide overviews and detailed step-by-step descriptions of newer and state-of-the-art methods utilized in the field. These include tools essential for forward and reverse genetic analysis, data mining and comparative genomics strategies, electron and fluorescence microscopy methods, automated imaging methods for worm behavioral analysis, functional genomics strategies, and methods for physiological analyses including somatic cell culture, toxicity assays, electrophysiology, and in vivo imaging of intracellular Ca^{2+} and pH using genetically encoded fluorescent indicator proteins. It is my hope that this book will be of use to both experts and newcomers to the field, not only as a step-by-step guide, but also as a roadmap to show what is possible with *C. elegans* and what has yet to be discovered.

Kevin Strange

References

1. Bloom, F. E. (2001) What does it all mean to you? *J. Neurosci.* **21,** 8304–8305.
2. Kitano, H. (2002) Looking beyond the details: a rise in system-oriented approaches in genetics and molecular biology. *Curr. Genet.* **41,** 1–10.
3. Kitano, H. (2002) Systems biology: a brief overview. *Science* **295,** 1662–1664.
4. Ideker, T., Galitski, T., and Hood, L. (2001) A new approach to decoding life: systems biology. *Annu. Rev. Genomics Hum. Genet.* **2,** 343–372.
5. Pennisi, E. (2003) Systems biology. Tracing life's circuitry. *Science* **302,** 1646–1649.
6. Sulston, J. E. and Horvitz, H. R. (1977) Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. *Dev. Biol.* **56,** 110–156.
7. Sulston, J. E., Schierenberg, E., White, J. G., and Thomson, J. N. (1983) The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **100,** 64–119.
8. White, J. G., Southgate, E., Thomson, J. N., and Brenner, S. (1986) The structure of the nervous system of the nematode *C. elegans*. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **314,** 1–340.

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Contributors

RAFFI V. AROIAN • *Section of Cell and Developmental Biology, University of California, San Diego, La Jolla, CA*

ROBERT J. BARSTEAD • *Oklahoma Medical Research Foundation, Oklahoma City, OK*

JEAN-LOUIS BESSEREAU • *Department of Biology, Ecole Normale Supérieure, Paris, France*

LARRY J. BISCHOF • *Section of Cell and Developmental Biology, University of California, San Diego, La Jolla, CA*

AVRIL COGHLAN • *Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Ireland; and Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, UK*

CHRISTOPHER J. CRONIN • *HHMI and Division of Biology, California Institute of Technology, Pasadena, CA*

M. WAYNE DAVIS • *Department of Biology, University of Utah, Salt Lake City, UT*

ZHAOYANG FENG • *Life Science Institute, University of Michigan, Ann Arbor, MI*

MICHAEL M. FRANCIS • *Department of Biology, University of Utah, Salt Lake City, UT*

MARC HAMMARLUND • *Department of Biology, University of Utah, Salt Lake City, UT*

TODD W. HARRIS • *Cold Spring Harbor Laboratory, Cold Spring Harbor, NY*

DANIELLE L. HUFFMAN • *Section of Cell and Developmental Biology, University of California, San Diego, La Jolla, CA*

HARALD HUTTER • *Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia, Canada*

REX A. KERR • *Division of Biological Sciences, University of California, San Diego, La Jolla, CA*

TODD LAMITINA • *Department of Anesthesiology, Vanderbilt University Medical Center, Nashville, TN*

ANDRES VILLU MARICQ • *Department of Biology, University of Utah, Salt Lake City, UT*

MICHAEL A. MILLER • *Department of Cell Biology, University of Alabama at Birmingham, Birmingham, AL*

DONALD G. MOERMAN • *Genome Sciences Centre, BC Cancer Agency, Vancouver, BC, Canada*

REBECCA MORRISON • *Department of Anesthesiology, Vanderbilt University Medical Center, Nashville, TN*

KEITH NEHRKE • *Nephrology Unit, Department of Medicine and Department of Pharmacology and Physiology, University of Rochester Medical Center, Rochester, NY*

VIDA PRAITIS • *Department of Biology, Grinnell College, Grinnell, IA*

ROCK PULAK • *Life Sciences Technology Group, Union Biometrica Inc., Holliston, MA*

WILLIAM R. SCHAFER • *Division of Biological Sciences, University of California, San Diego, La Jolla, CA*

JASON E. STAJICH • *Department of Molecular Genetics and Microbiology, Duke University, Durham, NC*

LINCOLN D. STEIN • *Cold Spring Harbor Laboratory, Cold Spring Harbor, NY*

KEVIN STRANGE • *Departments of Anesthesiology, Molecular Physiology and Biophysics, and Pharmacology, Vanderbilt University Medical Center, Nashville, TN*

LISA TIMMONS • *Department of Molecular Biosciences, University of Kansas, Lawrence, KS*

ROBBY M. WEIMER • *Cold Spring Harbor Laboratory, Cold Spring Harbor, NY*

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An Overview of *C. elegans* Biology

Kevin Strange

Summary

The establishment of *Caenorhabditis elegans* as a "model organism" began with the efforts of Sydney Brenner in the early 1960s. Brenner's focus was to find a suitable animal model in which the tools of genetic analysis could be used to define molecular mechanisms of development and nervous system function. *C. elegans* provides numerous experimental advantages for such studies. These advantages include a short life cycle, production of large numbers of offspring, easy and inexpensive laboratory culture, forward and reverse genetic tractability, and a relatively simple anatomy. This chapter will provide a brief overview of *C. elegans* biology.

Key Words: Genetics; anatomy; life cycle; laboratory culture.

1. Establishment of *Caenorhabditis elegans* as a "Model Organism"

The establishment of *Caenorhabditis elegans* as a model system for fundamental biological research began in 1963 with the efforts of Sydney Brenner, a molecular biologist then at the Medical Research Council Laboratory of Molecular Biology in Cambridge, England. In a letter to Max Perutz, the head of the laboratory, Brenner stated that, "it is widely realized that nearly all the 'classical' problems of molecular biology have either been solved or will be solved in the next decade" and that the "future of molecular biology lies in the extension of research to other fields of biology, notably development and the nervous system" (*1*).

The extraordinary successes that had been achieved at that time in defining the molecular bases of biological processes in bacteria suggested to Brenner that a similar approach would also be successful in more complex organisms. He told Perutz that he wanted to, "define the unitary steps of development using the techniques of genetic analysis." To tackle this problem, Brenner felt that he would

need to identify a metazoan animal that he could "microbiologize" and handle in much the same way as bacteria and viruses. Brenner concluded his letter by stating that he wanted to "tame a small metazoan organism to study development directly."

Despite criticism from other molecular biologists that his approach was too "biological," Brenner was asked to submit a formal proposal to the Medical Research Council. He did so in October 1963, proposing to study the genetics of differentiation in the nematode *Caenorhabditis briggsae**. In Brenner's opinion, *Caenorhabditis* provided numerous experimental advantages for identifying genes involved in development. The animal has a short life cycle, produces large numbers of offspring by sexual reproduction, and can be cultured easily in the laboratory. Sexual reproduction occurs by self-fertilization in hermaphrodite worms or by mating with males, which makes *Caenorhabditis* exceptionally useful for genetic studies. Self-fertilization allows homozygous worms to breed true and greatly facilitates the isolation and maintenance of mutant strains. It is also a handy feature if mutant animals are paralyzed or uncoordinated because reproduction does not require movement in order to find and mate with a male. Mating with males, however, is essential for moving mutations between strains. Finally, *Caenorhabditis* is a highly differentiated animal but is comprised of less than 1000 somatic cells and, therefore, provides a tractable system for studies of metazoan cellular function, development and differentiation.

Brenner concluded his proposal with the following statement: "To start with we propose to identify every cell in the worm and trace lineages. We shall also investigate the constancy of development and study its control by looking for mutants." *C. elegans* researchers have accomplished Brenner's goal of tracing the lineage of every nematode cell and identifying genes responsible for development. In 2002, Sydney Brenner shared the Nobel Prize in Physiology or Medicine with John Sulston and H. Robert Horvitz for their discovery of genes in *C. elegans* that regulate organ development and programmed cell death. However, the impact of *C. elegans* has extended well beyond the field of developmental biology. The extraordinary experimental power of the worm has been exploited to address a host of fundamental biological problems such as ageing, RNA-mediated gene silencing, cell cycle control, sensory physiology, and synaptic transmission.

2. *C. elegans* Biology

2.1. Natural History and Life Cycle

C. elegans is a member of the phylum Nematoda. Nematodes, or roundworms, are some of the most numerous and widespread of all animals and are

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C. elegans eventually replaced *C. briggsae* as the species of choice.

found in virtually all habitats. The phylum contains free-living species, as well as species that parasitize plants and other animals. In addition to its extraordinary utility as a model for basic biological research, detailed understanding of all aspects of *C. elegans* function has major economic and medical implications. Approximately half of the world's human population is infected with parasitic nematodes *(2)* and parasitic plant nematodes cause an estimated $80 billion in crop damage annually *(3)*.

Nematodes range in size from less than 1 mm to more than 35 cm in length. *C. elegans* is a free-living soil nematode about 1 mm long. The life strategy of *C. elegans* is well adapted for survival in soil environments where food and water availability, temperature, populations of predators, and many other variables can change constantly and dramatically. It is a voracious feeder and outgrows its competitors by producing large numbers of offspring and rapidly depleting local food resources.

Adult *C. elegans* are predominantly hermaphroditic with males making up approx 0.1% of wild-type populations. Self-fertilized hermaphrodites produce about 300 offspring, whereas male-fertilized hermaphrodites can produce more than 1000 progeny.

Under optimal laboratory conditions the average life span of *C. elegans* is 2–3 wk. The life cycle is rapid. At 25°C, embryogenesis, the period from fertilization until hatching, occurs in 14 h. Postembryonic development occurs in four larval stages (L1–L4) that last a total of about 35 h.

When food supply is limited, dauer larvae form after the second larval molt. Dauer larvae do not feed and have structural, metabolic, and behavioral adaptations that increase life span up to 10 times and aid in the dispersal of the animal to new habitats. Once food becomes available, dauer larvae feed and continue development to the adult stage *(4)*.

2.2. Laboratory Culture

The standard *C. elegans* laboratory strain is Bristol N2. Other strains are also used and offer certain experimental advantages. For example, the Bergerac strain exhibits a high rate of sponatenous mutation owing to the activity and high copy number of the Tc1 transposon *(5)*. The Hawaiian strain CB4856 possesses a uniformly high density of single-nucleotide polymorphisms that greatly facilitate genetic mapping *(6)*.

Culture of *C. elegans* in the laboratory is simple and relatively inexpensive *(7)*. Animals are typically grown in Petri dishes on agar seeded with a lawn of *Escherichia coli* as a food source. *C. elegans* can also be grown in mass quantities using liquid culture strategies and fermentor-like devices. Worm stocks are stored frozen in liquid nitrogen indefinitely with good viability. The ability to store *C. elegans* frozen dramatically simplifies culture strategies and reduces costs associated with handling and maintaining wild-type and mutant worm strains.

2.3. Anatomy

Like all nematodes, *C. elegans* has an unsegmented, cylindrical body that tapers at both ends. The body wall consists of tough collagenous cuticle underlain by hypodermis, muscles, and nerves. A fluid-filled body cavity or pseudocoel separates the body wall from internal organs. Body shape is maintained by hydrostatic pressure in the pseudocoel.

Newly hatched L1 larvae have 558 cells. Additional divisions of somatic blast cells occur during the four larval stages eventually giving rise to 959 somatic cells in mature adult hermaphrodites and 1031 in adult males. The lineage of somatic cells in *C. elegans* is largely invariant. This invariance combined with the ability to visualize by differential interference contrast microscopy cell division and development in living embryos, larvae, and adult animals has made it possible to describe the fate map or cell lineage of the worm *(8,9)*.

Despite the small cell number, *C. elegans* exhibits a striking degree of differentiation. Many physiological functions found in mammals have nematode analogs. This high degree of complexity and small total cell number provides a remarkably tractable experimental system for studies of differentiation, cell biology, and cell physiology. A detailed description of worm anatomy can be found online at the Center for *C. elegans* Anatomy (http://www.aecom.yu.edu/wormem/).

2.3.1. Skin

The worm "skin" or hypodermis is an epithelium that underlies the cuticle. Hypodermal cells secrete the cuticle, provide a substrate for cell and axon migration, and function as a storage site for lipids and other molecules *(10,11)*. The hypodermis is also involved in phagocytosis of apoptotic cells *(9,12)* and may have an osmoregulatory function *(13)*. Certain types of hypodermal cells function as blast cells and give rise postembryonically to new cell types, such as neurons *(9)*. Most hypodermal cells are multinucleate.

2.3.2. Muscle

C. elegans is a particularly powerful model for defining muscle physiology, structure, molecular biology, and development (reviewed in **refs.** *14* and *15*). The worm has a number of readily observable characteristics that allow rapid screening for mutations in genes involved in muscle function. Even mutations that cause severe locomotion defects can be studied readily and in detail because hermaphrodites self fertilize and locomotion is unnecessary for reproduction.

C. elegans possesses both striated and nonstriated muscles. Striated body wall muscles are the most numerous muscle cell type. They are arranged in longitudinal bands along the body wall and are responsible for locomotion. Nonstriated muscles are associated with the pharynx, intestine, anus, and the

hermaphrodite uterus, gonad sheath, and vulva. These muscles are responsible for pharyngeal pumping, defecation, ovulation and fertilization, and egg laying. Specialized nonstriated muscles are located in the tail of male worms and function in mating.

2.3.3. Nervous System

The nervous system of adult hermaphrodites contains 302 neurons and 56 glial and support cells. Males have 381 neurons and 92 glial and support cells. White et al. *(16)* have reconstructed and mapped the connectivity of the entire hermaphrodite nervous system using serial electron microscopy. Most of the differences between the male and hermaphrodite nervous system are found in the male tail, which plays an important role in mating.

The nervous system of *C. elegans* is generally thought of as being divided into the pharyngeal and central nervous systems (CNS). Twenty neurons innervate and regulate the activity of the pharynx. These neurons are connected to the CNS by two interneurons.

Neuronal processes of the CNS are oriented as bundles that run along the hypodermis in longitudinal cords or circumferential commissures. The ventral and dorsal nerve cords emanate from the circumpharyngeal nerve ring. Interneurons and motor neurons make up the ventral nerve cord. The dorsal nerve cord is formed by largely of ventral cord motor neuron axons that enter via commissures. Sense organs that respond to chemicals, temperature, mechanical force, and osmolality are located primarily in the head and tail.

An important feature of the *C. elegans* nervous system is that only three neurons, CANL, CANR, and M4, are required for viability under laboratory conditions. The CAN (excretory canal) neurons run along the excretory canals and may play an important role in regulating systemic salt and water balance. M4 is a pharyngeal motor neuron that controls isthmus peristalsis and, thus, controls feeding. The nonessential nature of most neurons for viability provides an enormous advantage for mutagenesis studies of nervous system function.

2.3.4. Kidney

The worm "kidney" consists of three cells types: the excretory cell, the duct cell, and the pore cell *(17)*. Destruction of any of these cells by laser ablation causes the animal to swell with fluid and die *(18)*.

The excretory cell is a large, H-shaped cell that sends out processes both anteriorly and posteriorly from the cell body. A fluid-filled excretory canal is surrounded by the cell cytoplasm. The basal pole of the cell faces the pseudocoel, whereas the apical membrane faces the excretory canal lumen. Gap junctions connect the excretory cell to the hypodermis, suggesting an interaction between the two cell types important for whole animal osmoregulation.

An excretory duct connects the excretory canal to the outside surface of the worm. The duct is formed from cuticle that is continuous with the animal's exoskeleton. A duct cell surrounds the upper two-thirds of the duct and a pore cell surrounds the lower third.

The excretory cell is a single-cell "epithelium" that appears to secrete salt, water, and waste products into the excretory canal. Duct cells may also play an important role in solute and water transport. The apical surface area of duct cells is greatly amplified by extensive invaginations and the cytoplasm is filled with mitochondria. Nelson et al. *(17)* have suggested that the duct cell may be involved in selective solute reabsorption. If this is the case, the nematode excretory and duct cells are analogous to the acini and ducts of mammalian secretory epithelia, such as the salivary gland, sweat glands, and pancreas.

2.3.5. Digestive Tract

The digestive tract of *C. elegans* consists of a pharynx, intestine, and rectum. *C. elegans* is a filter feeder and the pharynx is a muscular organ that pumps food into the pharyngeal lumen, grinds it up, and then moves it into the intestine. The pharynx is formed from muscles cells, neurons, epithelial cells, and gland cells *(19)*.

A pharyngeal–intestinal valve connects the pharynx to the intestine. Twenty epithelial cells with extensive apical microvilli form the main body of the intestine *(20)*. Intestinal epithelial cells secrete digestive enzymes and absorb nutrients. The intestine functions as one of the major storage organs in the body. Intestinal cells are filled with numerous granules that are likely to contain lipids, proteins, and carbohydrates. The intestine also produces yolk proteins and secretes them into the pseudocoel where they are then taken up by oocytes *(21,22)*.

The rectum is made up of five epithelial cells and is connected to the intestine via the intestinal–rectal valve. A sphincter muscle wraps around the valve and controls its opening. The sphincter muscle, an anal depressor muscle, and two muscle cells that wrap around the posterior intestine control defecation.

2.3.6. Gonad

The gonad of adult hermaphrodites consists of two identical U-shaped arms connected via spermatheca to a common uterus *(23,24)*. The gonad arms are surrounded by thin epithelial cells termed "sheath cells." The distal portion of each arm contains germline nuclei that give rise to sperm and oocytes. Each nucleus is surrounded by cytoplasm and an incompletely formed plasma membrane. The nuclei in turn are arranged in a monolayer that surrounds a central core of cytoplasm.

The germline nuclei population is maintained by mitosis occurring in the distal-most tip of each gonad arm. As germ cells progress down the arm, they exit the mitotic cell cycle and undergo meiosis. Formation of an intact germ cell plasma membrane, a process referred to as cellularization, occurs in the proximal arm of the gonad. During the fourth larval stage, germ cells differentiate into approx 150 spermatids per gonad arm. These spermatids develop into mature sperm that are stored in the spermatheca.

In adult worms, germ cells differentiate into oocytes. Oocytes are arranged in the proximal gonad arm in a single-file fashion. Once formed, oocytes undergo oogenesis, which is a period of intense biosynthetic activity and rapid and massive oocyte growth.

Oocytes remain in diakinesis of prophase I until they reach the most proximal position in the gonad arm. During the late stage of oogenesis, an oocyte located immediately adjacent to the spermatheca undergoes meiotic maturation. Within 5–6 min after maturation is initiated, the oocyte is ovulated into the spermatheca where it is fertilized. Completion of the meiotic divisions occurs in the uterus and is followed by embryogenesis. In a mature hermaphrodite, ovulation occurs once every 20–40 min. Unmated hermaphrodites produce approx 300 progeny over a 3-d period when grown under standard laboratory conditions *(24)*.

The testis, seminal vesicle, and vas deferens form the male gonad *(25,26)*. The testis is U-shaped and is formed from two distal tip cells that surround the germ cells. As in the hermaphrodite gonad, germ cell nuclei at the distal end of the testis undergo mitosis. Germ nuclei farther away enter prophase of meiosis I. Spermatogenesis begins in the proximal end of the testis.

The seminal vesicle is formed by 20 secretory cells. Spermatocytes enter the vesicle and undergo two meiotic divisions to form mature spermatozoa. Spermatozoa are stored in the seminal vesicle. The vas deferens is comprised of 30 cells and functions as a valve that controls the release of sperm during ejaculation.

3. Online Access to *C. elegans* Biology

The "worm community" is well-known for its open sharing of data and reagents. As noted earlier, numerous reagents including mutant and transgenic worm strains, cosmid, and yeast artificial chromosome clones, and expressed sequence tags (EST) clones are freely available from public resources (*see* **Table 1**). In addition, an extraordinary wealth of data on *C. elegans* is available online. Indeed, the worm community was an early pioneer in the use of the Internet for electronic data sharing. WormBase (http://www.wormbase.org/) is a particularly noteworthy database. It provides an exhaustive catalog of worm biology including identification of all known and predicted worm genes. Gene descriptions include genome location, mutant and RNAi phenotypes, expression patterns, microarray data, gene ontology, mutant alleles, and BLAST matches.

Table 1
Publicly Available Online *C. elegans* Databases and Resources

Site	Information and services available	URL
Anatomy		
• The Center for *C. elegans* Anatomy	Aid and train investigators in *C. elegans* electron microscopy	http://www.aecom.yu.edu/wormem/
• WormAtlas	Online database of worm anatomy, anatomical methods, and cell function and identification	http://www.wormatlas.org/
• Wormimage	Online database of *C. elegans* electron micrographs and associated data	http://www.wormimage.org/
Genomics		
• WormBase	Repository of information on genetics, genomics, and biology of *C. elegans* including gene sequence, gene expression patterns, RNA_i phenotypes, genetic maps, and worm anatomy	http://www.wormbase.org/
• ORFeome project	Database of *C. elegans* ORFs	http://worfdb.dfci.harvard.edu/
Gene knockout		
• The *C. elegans* Gene Knockout Consortium	Produce null alleles of all known *C. elegans* genes; knockout animals available through the *Caenorhabditis* genetics center	http://elegans.bcgsc.bc.ca/knockout.shtml
• Japanese National Bioresource Project	Produce null alleles of all known *C. elegans* genes; provide knockout animals to investigators	http://shigen.lab.nig.ac.jp/c.elegans/index.jsp
Protein–protein interactions		
• Vidal lab INTERACTome Project	Genome-scale protein–protein interaction mapping	http://vidal.dfci.harvard.edu/main_pages/interactome.htm

Gene expression patterns

- C. elegans Gene Expression Consortium — Microarray, SAGE, and GFP analysis of gene expression patterns — http://elegans.bcgsc.ca/home/ge_consortium.html /
- The Hope Laboratory Expression Pattern Database — lacZ and GFP reporter expression database — http://129.11.204.86:591/default.htm

Worm reagents[a]

- Caenorhabditis Genetics Center — Collect, maintain, and distribute worm strains; maintain a C. elegans bibliography — http://biosci.umn.edu/CGC/CGChomepage.htm
- MRC geneservice — Genome-wide RNAi library ORF clones — http://www.geneservice.co.uk/products/home/
- Addgene — Fire Lab C. elegans Vector Kit: contains 288 vectors for lacZ and/or GFP expression, RNAi feeding, tagging C. elegans exons and introns with GFP, RNAi hairpin sequences, etc. — http://www.addgene.org/pgvec1?f=c&cmd=showcol&colid=1
- Open biosystems — ORF clones RNAi clones — http://www.openbiosystems.com/index.php
- Sanger Institute — Provide cosmid and YAC clones to investigators — http://www.sanger.ac.uk/Projects/C_elegans/

Miscellaneous

- Caenorhabditis elegans WWW Server — Links to many useful sites including an online catalog of methods — http://elegans.swmed.edu/
- Textpresso — Search engine for C. elegans literature — http://www.wormimage.org/
- WormBook — Online reviews of C. elegans biology and methodology — http://www.wormbook.org/
- A Caenorhabditis elegans Survival Kit — Links to many useful sites — http://members.tripod.com/C.elegans/c_elegans_Introduction_Value_to_Biology_Medicine.htm

[a]Check WormBase for latest information on ordering genomic clones.
GFP, green fluorescent protein; MRC, Medical Research Council; ORF, open reading frame; SAGE, serial analysis of gene expression; YAC, yeast artificial chromosome.

Acknowledgments

References

1. Brenner, S. (1988) Foreward. In: *The Nematode* Caenorhabditis elegans, (Wood, W. B. and the Community of *C. elegans* Researchers, eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. ix–xiii.
2. Markell, E. K. and Voge, M. (1981) *Medical Parasitology,* W. B. Saunders and Company, Philadelphia, PA.
3. Sasser, J. N. and Freckman, D. W. (1987) A world perspective of nematology: the role of the society. In: *Vistas on Nematology,* (Veech, J. A. and Dickson, D. W., eds.), Society of Nematologists, Hyattsville, MD, pp. 7–14.
4. Smeal, T. and Guarente, L. (1997) Mechanisms of cellular senescence. *Curr. Opin. Genet. Dev.* **7,** 281–287.
5. Moerman, D. G. and Waterston, R. H. (1984) Spontaneous unstable *unc-22* IV mutations in *C. elegans* var. Bergerac. *Genetics* **108,** 859–877.
6. Wicks, S. R., Yeh, R. T., Gish, W. R., Waterston, R. H., and Plasterk, R. H. (2001) Rapid gene mapping in *Caenorhabditis elegans* using a high density polymorphism map. *Nat. Genet.* **28,** 160–164.
7. Lewis, J. A. and Fleming, J. T. (1995) Basic culture methods. In: Caenorhabditis elegans: *Modern Biological Analysis of an Organism,* (Epstein, H. F. and Shakes, D. C., eds.), Academic Press, New York, NY, pp. 4–29.
8. Sulston, J. E. and Horvitz, H. R. (1977) Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. *Dev. Biol.* **56,** 110–156.
9. Sulston, J. E., Schierenberg, E., White, J. G., and Thomson, J. N. (1983) The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **100,** 64–119.
10. White, J. (1988) The anatomy. In: *The Nematode* Caenorhabditis elegans, (Wood, W. B. and the Community of *C. elegans* Researchers, eds,), Cold Srping Harbor Press, Cold Spring Harbor, NY, pp. 81–122.
11. Hedgecock, E. M., Culotti, J. G., Hall, D. H., and Stern, B. D. (1987) Genetics of cell and axon migrations in *Caenorhabditis elegans*. *Development* **100,** 365–382.
12. Robertson, A. and Thomson, N. (1982) Morphology of programmed cell death in the ventral nerve cord of *Caenorhabditis elegans* larvae. *J. Embryol. Exp. Morphol.* **67,** 89–100.
13. Petalcorin, M. I., Oka, T., Koga, M., et al. (1999) Disruption of *clh-1*, a chloride channel gene, results in a wider body of *Caenorhabditis elegans*. *J Mol. Biol.* **294,** 347–355.
14. Moerman, D. G. and Fire, A. (1997) Muscle: structure, function, and development. In: C. elegans *II,* (Riddle, D. L., Blumenthal, T., Meyer, B. J., and Priess, J. R., eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 417–470.

15. Waterston, R. H. (1988) Muscle. In: *The Nematode* Caenorhabditis elegans (Wood, W. B. and the Community of *C. elegans* Researchers, eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 281–335.
16. White, J. G., Southgate, E., Thmson, J. N., and Brenner, S. (1986) The structure of the nervous system of the nematode *C. elegans*. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **314,** 1–340.
17. Nelson, F. K., Albert, P. S., and Riddle, D. L. (1983) Fine structure of the *Caenorhabditis elegans* secretory-excretory system. *J. Ultrastruct. Res.* **82,** 156–171.
18. Nelson, F. K. and Riddle, D. L. (1984) Functional study of the *Caenorhabditis elegans* secretory-excretory system using laser microsurgery. *J. Exp. Zool.* **231,** 45–56.
19. Albertson, D. G. and Thomson, J. N. (1976) The pharynx of *Caenorhabditis elegans*. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **275,** 299–325.
20. Leung, B., Hermann, G. J., and Priess, J. R. (1999) Organogenesis of the *Caenorhabditis elegans* intestine. *Dev. Biol.* **216,** 114–134.
21. Grant, B. and Hirsh, D. (1999) Receptor-mediated endocytosis in the *Caenorhabditis elegans* oocyte. *Mol. Biol. Cell* **10,** 4311–4326.
22. Hall, D. H., Winfrey, V. P., Blaeuer, G., et al. (1999) Ultrastructural features of the adult hermaphrodite gonad of *Caenorhabditis elegans*: relations between the germ line and soma. *Dev. Biol.* **212,** 101–123.
23. Hubbard, E. J. and Greenstein, D. (2000) The *Caenorhabditis elegans* gonad: a test tube for cell and developmental biology. *Dev Dyn.* **218,** 2–22.
24. Hirsh, D., Oppenheim, D., and Klass, M. (1976) Development of the reproductive system of *Caenorhabditis elegans*. *Dev. Biol.* **49,** 200–219.
25. Klass, M., Wolf, N., and Hirsh, D. (1976) Development of the male reproductive system and sexual transformation in the nematode *Caenorhabditis elegans*. *Dev. Biol.* **52,** 1–18.
26. Ward, S. and Carrel, J. S. (1979) Fertilization and sperm competition in the nematode *Caenorhabditis elegans*. *Dev. Biol.* **73,** 304–321.

2

Comparative Genomics in *C. elegans, C. briggsae,* and Other *Caenorhabditis* Species

Avril Coghlan, Jason E. Stajich, and Todd W. Harris

Summary

The genome of the nematode *Caenorhabditis elegans* was the first animal genome sequenced. Subsequent sequencing of the *Caenorhabditis briggsae* genome enabled a comparison of the genomes of two nematode species. In this chapter, we describe the methods that we used to compare the *C. elegans* genome to that of *C. briggsae*. We discuss how these methods could be developed to compare the *C. elegans* and *C. briggsae* genomes to those of *Caenorhabditis remanei*, *C.* n. sp. represented by strains PB2801 and CB5161, among others *(1)*, and *Caenorhabditis japonica*, which are currently being sequenced.

Key Words: Nematode genomes; *Caenorhabditis* genomes; comparative genomics; genome evolution; nematodes.

1. Introduction

The *Caenorhabditis elegans* and *Caenorhabditis briggsae* genomes were the first pair of genomes from the same animal genus to be sequenced *(1a,2)*. Comparison of their gene content and chromosomal structure has provided insight into the evolution of nematodes. In the last 2 yr, methods have been developed that exploit comparisons between multiple species to predict genes and regulatory elements, and to study genome evolution. We discuss how these methods could be used to compare the *C. elegans* and *C. briggsae* genomes to those of *Caenorhabditis remanei*, *C.* sp. PB2801, and *Caenorhabditis japonica*, which are currently being sequenced (**Fig. 1; ref. *3***).

2. Predicting Genes and Comparing Gene Structure in Related Genomes
2.1. Predicting Genes in Related Genomes

There were 19,735 protein-coding genes annotated in the *C. elegans* genome in a recent release of WormBase (WS140 *[4]*). These annotations have been
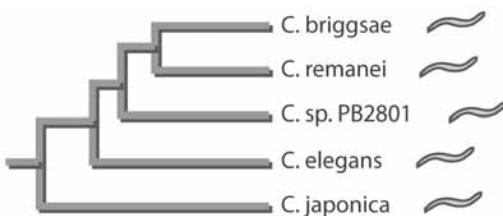
Fig. 1. The phylogenetic relationship of *Caenorhabditis elegans* and *Caenor-habditis briggsae* to *Caenorhabditis remanei*, *C.* sp. PB2801, and *Caenorhabditis japonica*, whose genomes are being sequenced. This figure is based on the phylogeny found by Kiontke et al. *(19)*.

extensively tested and improved using computer programs and experiments. In fact, more than 17,000 *C. elegans* genes have been partially or fully confirmed by mRNA and other experiments *(5)*. The *C. briggsae* genome is similar in size to that of *C. elegans*, approx 100 Mb, and has a similar number of genes, approx 20,000 genes *(2)*. It will be interesting to see whether *C. remanei*, *C.* sp. PB2801, and *C. japonica* also have a similar number of genes and genome size.

The most common programs used to predict protein-coding genes are those that predict genes based on sequence alone, known as "*de novo* gene predictors." Two *de novo* gene predictors that have been tuned for nematode genomes are Genefinder (P. Green, unpublished) and Fgenesh *(6)*, which were used to predict genes in the *C. briggsae* genome *(2)*. Both Genefinder and Fgenesh are relatively accurate. Genefinder predicts 48% of known *C. elegans* genes and 81% of known exons exactly right, whereas Fgenesh predicts 51% of known *C. elegans* genes and 88% of known exons correctly *(7,8)*.

An advance in accuracy has been enabled by the development of gene predictors that use information from the alignment of the target genome to a second genome *(9)*. These exploit the fact that protein-coding exons are better conserved over evolutionary time than nonfunctional intergenic or intronic regions. One program that uses this approach is TWINSCAN *(10)*, which was used to predict genes in the *C. briggsae* genome using alignments to *C. elegans* *(2)*. TWINSCAN is more accurate than Fgenesh or Genefinder. Using alignments to the *C. briggsae* genome, TWINSCAN predicts 60–63% of known *C. elegans* genes correctly, and 86–90% of known exons *(8)*. This is partly because of the use of cross-species alignments, but is also because of more accurate prediction of intron length and of introns with noncanonical splice sites *(8)*. The optimal evolutionary divergence for TWINSCAN is approx 90–95%, which is less than the divergence between *C. elegans* and *C. briggsae* *(8)*. Thus, TWINSCAN will probably produce more accurate predictions for less divergent pairs of nematodes,

such as *C. briggsae* and *C. remanei* (**Fig. 1**). Gene predictors that use alignments between more than two species have begun to appear. Multispecies alignments are used by the program EXONIPHY to predict exons *(11)*, and by Evogene to predict genes *(12)*. These programs will be useful for producing accurate gene predictions for *C. remanei*, *C.* sp. PB2801, and *C. japonica*, as well as for improving *C. elegans* and *C. briggsae* annotation.

Wei et al. *(8)* recently demonstrated the potential of interspecies comparisons for improving *C. elegans* annotation. They used TWINSCAN to predict 265 open reading frames in the *C. elegans* genome that did not overlap existing gene predictions. They successfully cloned and sequenced 146 of these open reading frames, thereby finding evidence for 146 previously unrecognized genes. Interspecies comparisons can also identify unrecognized exons that are part of known genes. For example, by comparing the *C. elegans* epidermal growth gene *lin-3* to its *C. briggsae* ortholog, Liu et al. *(13)* predicted an alternatively spliced 5' exon, which they confirmed experimentally. By aligning *C. elegans* genes to the *C. briggsae* genome sequence, Stein et al. *(2)* suggested that there may be more than 1200 unrecognized exons in known *C. elegans* genes. Multispecies comparisons will help to distinguish how many of these putative exons are real. If a putative exon seems to be conserved in *C. remanei*, *C.* sp. PB2801, and *C. japonica*, as well as in *C. briggsae* and *C. elegans*, it is likely to be real. One caveat to remember is that some gene structures have probably changed since the species diverged. For example, changes in splice sites have led to different alternative splicing of the CPEB genes *fog-1* and *cpb-2* in the five *Caenorhabditis* species *(14)*.

A major challenge in gene prediction is annotating species-specific genes, because we cannot use alignments between species to predict them, or to support predictions. Stein et al. *(2)* predicted approx 1000 *C. elegans*-specific genes and approx 110 *C. elegans*-specific gene families. Some of these may simply be genes that have evolved very rapidly. If this is true, it may be possible to identify their orthologs in other species by using synteny information, or by using sensitive homology search methods, such as profile-hidden Markov models *(15)*. However, some of the putative species-specific genes are probably novel genes that have been generated since the species diverged *(16)*. Even though such genes are difficult to predict, they are of great interest because they may be involved in species-specific adaptations *(17)*.

2.2. Comparing Gene Structure in Related Genomes

The five *Caenorhabditis* genomes will be a treasure trove for studying the evolution of gene structure. There are many unsolved questions about the evolution of intron–exon structure and splicing patterns. For example, it is not known how introns are gained or lost *(14,18)*. The *Caenorhabditis* genomes

will provide fertile ground for studying this question, because approx 9% of *C. elegans* and *C. briggsae* introns are species-specific *(2)*. To distinguish whether the *C. elegans*-specific introns were gained by *C. elegans* or lost from *C. briggsae*, an outgroup species is needed. The *C. japonica* genome will provide such an outgroup (**Fig. 1**). For example, Kiontke et al. *(19)* cloned the gene for the largest subunit of RNA polymerase II from 10 *Caenorhabditis* species. By comparing their intron–exon structures, they estimated that there have been up to 19 intron losses and up to 15 gains in this gene since the species diverged. Similarly, Cho et al. *(14)* cloned five genes from six *Caenorhabditis* species, and also found loss to be more common than gain. It will be exciting to extend these studies to all 20,000 genes in the *Caenorhabditis* genomes. In particular, examination of introns that have been lost or gained very recently—those that are specific to just one species or strain—may help uncover the mechanisms of intron loss and gain. It may also reveal whether losses and gains affect gene expression or function, and could even be adaptive *(20)*.

3. Studying Orthologs, Paralogs, and Gene Families
3.1. Identifying Orthologs and Paralogs Between Genomes

Orthologs are genes in different species that evolved from a common ancestral gene by speciation. In contrast, paralogs are genes that originated by duplication within a genome. Stein et al. *(2)* identified 11,255 *C. briggsae–C. elegans* one-to-one orthologs, by identifying *C. briggsae–C. elegans* gene pairs that were each other's top BLASTP match *(21)*. To avoid contaminating the ortholog set with paralogs, ortholog pairs also had to have BLASTP *E*-values more than 10^5 lower (more significant) than the next-best match. This approach can miss one-to-one orthologs that have evolved rapidly or that belong to gene families, but ensures a high-confidence set of ortholog pairs. Stein et al. *(2)* identified an additional 900 ortholog pairs using conserved gene order. The final set of 12,155 one-to-one orthologs included approx 60–65% of the *C. elegans* and *C. briggsae* gene sets. The remaining 35–40% of the genes in each species are species-specific genes, or have multiple orthologs in the other species.

Additional methods to identify orthologs include InParanoid *(22)*, which identifies one-to-one, one-to-many, and many-to-many orthologs between two genomes based on BLASTP similarity scores, providing a confidence measure for each ortholog assignment. InParanoid identifies 12,858 *C. briggsae–C. elegans* ortholog groups (*see* http://inparanoid.cgb.ki.se/). On the other hand, OrthoMCL *(23)* identifies one-to-one, one-to-many, and many-to-many orthologs between multiple genomes, by using the Markov Cluster Algorithm (MCL) *(24)*, which groups related sequences into clusters. OrthoMCL distinguishes recent paralogs from orthologs by identifying sequences that have closer BLASTP matches within a species than between species.

The most accurate method of identifying one-to-one, one-to-many, and many-to-many orthologs between multiple genomes is to build phylogenetic trees *(25)*. By building a phylogenetic tree of the *sra* chemosensory receptor family, Stein et al. *(2)* showed that assigning one-to-one orthologs using mutual best BLASTP matches leads to false-positive and false-negative assignments approx 15% of the time. However, Stein et al. could not use phylogenetic trees to identify all *C. briggsae–C. elegans* orthologs, because they lacked sequences from an outgroup to root the trees. The *C. japonica* genome will provide an outgroup **(Fig. 1)**, and so will allow *C. briggsae–C. elegans* orthologs to be identified accurately.

3.2. Identifying Gene Families

Gene duplication provides a substrate for new gene functions *(26)*, so that lineage-specific duplications often trigger lineage-specific adaptations. In *C. elegans* several gene families have undergone lineage-specific expansions, including subfamilies of the seven-transmembrane (7TM) proteins, some of which function as chemoreceptors *(27)*. These 7TM gene duplications occurred in the *C. elegans* genome after divergence from *C. briggsae*, in two specific chemoreceptor families *(2,28)*.

The TRIBE-MCL program can be used to identify gene families *(29)*. It builds a graph of all proteins based on sequence similarity, where each protein is a node in the graph, and the distance between nodes is a function of the similarity between proteins. Most pairs of nodes are not connected by edges, so the graph is sparse. The TRIBE-MCL program finds clusters of similar nodes based on the internode distances, using the MCL *(24)*. MCL is an algorithm that applies a series of expansions and contractions to the graph in order to identify robust clusters. MCL differs from simpler clustering methods, such as single-linkage clustering, which builds a cluster of proteins by finding all interconnected nodes. A limitation of single-linkage clustering is its tendency to build clusters that are too heterogeneous, because a single protein domain shared among otherwise unrelated proteins serves as a connector between all the nodes.

To identify gene families that are significantly larger in one species than another, TRIBE-MCL can be used to build gene families using the entire protein sets from the two species. A χ^2 test can then be used to identify families that have a significantly greater copy number in one species than in the other. Applying this approach to multiple nematode genomes will allow us to identify families that have contracted or expanded within each lineage. For example, in the genomes of parasitic nematodes such as *Brugia malayi (30)* one might expect gene families necessary for a free-living lifestyle to have contracted and families related to evasion of the host immune system to have expanded.

Comparison of the *C. elegans* and *C. briggsae* genomes revealed that several families have significantly expanded within each lineage *(2)*. **Table 1** lists the 26

Table 1
The 26 Largest Gene Families in *Caenorhabditis elegans* and *Caenorhabditis briggsae*[a]

Cluster	*C. briggsae*	*C. elegans*	Result of χ^2 test	Cluster function
0	299	275	Not significant	Protein kinase
1	268	284	Not significant	Transcription factor, zinc finger
2	224	266	Not significant	7TM subfamily 1
3	163	312	Significant	7TM subfamily 2
4	230	184	Not significant	EGF-like
5	222	19	Significant	Unknown function
6	122	116	Not significant	BTB/POZ
7	97	141	Significant	Serpentine receptor (some are pseudogenes)
8	14	211	Significant	F-box/FTH
9	88	137	Significant	Lectin
10	93	111	Not significant	Unknown: DUF216
11	102	96	Not significant	Cuticle collagen
12	80	117	Not significant	Tyrosine specific protein phosphatase
13	103	94	Not significant	Neurotransmitter-gated ion-channel ligand binding domain
14	97	89	Not significant	Myosin
15	94	92	Not significant	G protein β WD-40 repeat
16	91	90	Not significant	UDP-glucoronosyl/UDP-glucosyl transferase
17	87	94	Not significant	Protein kinase
18	96	80	Not significant	Transcription factor, zinc finger
19	88	79	Not significant	RNA binding protein
20	73	82	Not significant	Cytochrome P450
21	78	70	Not significant	Cuticle collagen
22	72	70	Not significant	Ras GTPase
23	62	80	Not significant	Lectin
24	74	67	Not significant	Rhodopsin-like GPCR superfamily (serpentine receptor)
25	10	130	Significant	F-box

[a]Found using TRIBE-MCL (**29**). For each family, a χ^2 test was used to test whether the size of the family is significantly different in the two species.

7TM, seven transmembrane; EGF, epidermal growth factor; BTP, bric-a-brac, tramtrack, and Broad-Complex proteins; POZ, Pox virus and Zine finger; FTH, FOG-2 homology domain; WD, G-β repeat domain (Trp-Asp dipeptide); GPCR, G protein-coupled receptor.

largest families in each species. One of the most striking examples of *C. elegans*-specific expansion is a subfamily of 7TM chemoreceptors. A detailed analysis of this family showed that the expansion was caused by a series of local tandem duplications, presumably through unequal crossing over *(28)*. Many of the other apparent gene family expansions were actually owing to duplications of pseudogenes, rather than to duplication of functional genes. For example, cluster 7 in **Table 1** initially appeared to be because of an expansion of serpentine and rhodopsin-like G protein-coupled receptor genes, but many of these genes have since been identified as pseudogenes. Identifying significantly expanded families in the *C. elegans* and *C. briggsae* lineages is a very active area of research. It is not known how important these expansions are to the current lifestyle of each species. For example, it will be important to determine whether recently acquired chemoreceptors provide additional sensing for *C. elegans*, perhaps by binding additional ligands or by using combinations of receptors.

To more accurately identify families that have expanded recently, sophisticated models of gene family evolution are now being developed, incorporating the time since speciation and the rate of gene duplication. The best approach for identifying lineage-specific expansions is to use phylogenetic trees, including family members from more than two species (Hahn, M. W., De Bie, T., Stajich, J. E., Nguyen, C., and Cristianini, N., manuscript in prep.).

4. Computational Prediction of Regulatory Elements

The discovery of sequence elements that control the temporal and spatial expression of genes is a difficult but exciting area of comparative genomics. The small size of intergenic regions in *C. elegans* and *C. briggsae*, which are generally less than 1.5 kb, helps in this search. Furthermore, *C. elegans* and *C. briggsae* seem to be at an ideal evolutionary distance for identifying functionally important noncoding sequences. Webb et al. *(31)* compared 142 orthologous intergenic regions in *C. elegans* and *C. briggsae*, and found a mosaic pattern with regions of high similarity scattered among longer nonalignable regions. They suggested that the regions of high similarity might contain regulatory sequences.

A standard approach for finding candidate regulatory sequences is to align 0.5–2.0 kb upstream of orthologous *C. elegans* and *C. briggsae* genes. An important step is to repeat-mask the sequences first—to reduce the amount of spurious alignments. The upstream sequences can then be aligned using a local alignment algorithm, such as BLASTZ *(32)*, or a global alignment algorithm, such as GLASS *(33)*, or both. The alignment is scanned for conserved noncoding sequences (CNSs): long stretches of high-sequence identity, for example, of more than 70% identity over more than 50 bp. This approach has been successfully used to find regulatory sequences that control *C. elegans* pharyngeal development **(Fig. 2; ref. *34*)** and vulval expression *(35)*.
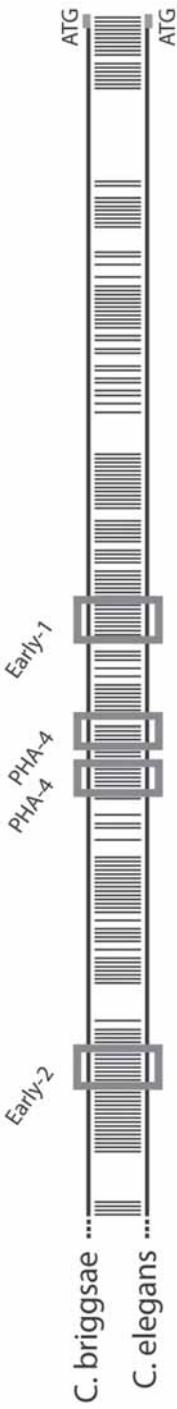
Fig. 2. A conserved noncoding sequence (CNS) found by Gaudet et al. (*34*) upstream of the *Caenorhabditis elegans* pharyngeal gene *K07C11.4* and its *Caenorhabditis briggsae* ortholog. Gaudet et al. identified four conserved transcription factor binding sites in this CNS (shown as gray boxes).

Identifying transcription factor binding sites within CNSs is difficult, because they can be as short as 6 bp. A common approach is to search for matches to known transcription factor binding sites in the TRANSFAC database *(36)*. Alternatively, if several *C. elegans* genes are probably regulated by the same transcription factor, one can search for short motifs that are overrepresented in CNSs in the upstream regions of the *C. elegans* genes and their *C. briggsae* orthologs *(34,35,37)*. This approach has the advantage that it can identify novel binding sites that are absent from TRANSFAC. For example, Gaudet et al. *(34)* discovered novel transcription factor binding sites in *C. elegans* genes expressed in the pharynx, by using the program ImProbizer *(38)* to find motifs that are unexpectedly frequent in their upstream regions (**Fig. 2**). Many other programs exist for identifying potential transcription factor binding sites in CNSs. Tompa et al. *(39)* recently assessed the accuracy of 13 motif-discovery programs in identifying known transcription factor binding sites. One of the most successful programs was Weeder *(40)*, which counts how often all possible motifs up to a certain length occur in the input sequences, and identifies overrepresented motifs that are well conserved.

GuhaThakurta et al. *(37)* tested the hypothesis that most *C. elegans* regulatory sequences can be found by searching within CNSs in orthologous *C. briggsae–C. elegans* upstream regions. They aligned 2 kb upstream of 33 *C. elegans* muscle-expressed genes to the upstream regions of their *C. briggsae* orthologs, using both local and global alignment algorithms. They then scanned the alignments to find CNSs with more than 65% identity over more than 50 bp. For some *C. elegans* genes the CNSs contained 55% of predicted muscle transcription factor binding sites, but for other genes they contained just 6% of predicted sites. GuhaThakurta et al. suggested that errors in the alignments used to identify CNSs might explain why CNSs do not contain all *C. elegans* transcription factor binding sites. Another reason is that the number of binding sites, their positions and sequences probably differ between *C. elegans* and *C. briggsae*. For example, the *lin-48* gene has a conserved expression pattern in *C. elegans* and *C. briggsae*, but the *C. elegans* upstream region contains two binding sites for the transcription factor EGL-38, whereas the *C. briggsae* promoter contains just one site *(41)*. Furthermore, the *C. elegans* and *C. briggsae* EGL-38 binding sites have diverged in sequence, so that the *C. briggsae* site cannot compensate for mutations in the *C. elegans* sites.

Comparison of the upstream regions of the *C. elegans* and *C. briggsae lin-3* genes to those of their *C. remanei* and *C.* sp. PB2801 orthologs suggests that multi-species comparisons will help to distinguish functionally important conserved sequences from sequences that have been conserved between *C. elegans* and *C. briggsae* by chance *(3)*. The addition of the *C. remanei* and *C.* sp. PB2801 genomes should also help to locate transcription factor binding sites within CNSs.

5. Detecting Syntenic Blocks and Chromosomal Rearrangements

5.1. Creating Whole Genome Alignments

Comparative studies of genome structure and evolution, and of conserved protein-coding and noncoding regions, typically rely on high-quality nucleotide-level alignments. Most pairwise genome alignments begin with a genome-wide set of local alignments—which are then stitched together into non-overlapping blocks that can be used for an analysis of synteny.

Among the many alignment algorithms available, the WABA *(42)* and BLASTZ *(32)* algorithms have been particularly effective at aligning the *C. elegans* and *C. briggsae* genomes *(2)*. Unlike algorithms tuned for aligning protein-coding regions, WABA and BLASTZ can effectively align regions of genomes that are evolving neutrally. WABA generates a set of local alignments between genomes, and discriminates between coding and noncoding regions, and between "strong" and "weak" alignments. As such, WABA is useful for identifying conserved coding regions between genomes. WABA alignments between *C. elegans* and *C. briggsae* cover 65% of their genomes *(2)*. Surprisingly, alignments between *C. elegans* and *C. briggsae* are as common in introns and intergenic regions as in coding regions, so are useful for locating potential regulatory sequences *(31)*. WABA alignments between *C. elegans* and *C. briggsae* can be downloaded from WormBase (http://www.wormbase.org) *(4)*.

The BLASTZ algorithm *(32)* is derived from gapped BLAST *(21)*, with enhancements for aligning long sequences. In initial tests, WABA and BLASTZ performed comparably at aligning the *C. elegans* and *C. briggsae* genomes, resulting in 65 and 56% non-overlapping coverage, respectively. Generating whole genome alignments between species using BLASTZ or WABA requires considerable computational resources and time. Nonetheless, alignments can be generated between a small set of regions and a target genome quickly and easily on modest hardware. For example, upstream regions of *C. elegans* genes could be rapidly aligned to a newly sequenced genome to search for candidate regulatory sequences.

New methods have recently been developed to align multiple genomes simultaneously, such as MULTIZ *(43)*. MULTIZ uses pairwise alignments between two genomes to guide subsequent alignments with other genomes. MULTIZ offers several advantages over pairwise alignments, including independence from the reference genome (*C. elegans* in the case of *Caenorhabditis* species). Pairwise and multiple alignments between the *C. elegans*, *C. briggsae*, *C. remanei*, *C.* sp. PB2801, and *C. japonica* genomes will be made available from WormBase *(4)* in the near future.

5.2. Detecting Syntenic Blocks

Synteny is the colinearity of genes between species. For whole genome analyses, this definition is often extended to include colinearity of segments of chro-

mosomes, not just of those regions containing genes. Analyses of synteny typically rely on nucleotide-level alignments or on sets of anchor points between two genomes. Nucleotide-level alignments usually require some postprocessing in order to generate a set of syntenic blocks. This is because a region in one genome may have multiple alignments that map to several different regions in the second genome. Furthermore, an alignment may be much shorter than the syntenic block that contains it—if the alignment algorithm halted prematurely in a poorly conserved region. No hard and fast rules exist for deriving syntenic blocks from nucleotide-level alignments; the process usually needs to be modified according to the degree of similarity between genomes.

The *C. briggsae–C. elegans* synteny analysis illustrates one method of postprocessing a set of whole genome nucleotide-level alignments to find larger syntenic blocks (*2*). First, adjacent alignments that had the same WABA status (which can be "weak," "strong," or "coding") were merged (**Fig. 3**). Second, *C. elegans* blocks that contained nucleotide-level alignments to more than five *C. briggsae* blocks, and *C. briggsae* blocks that contained alignments to more than five *C. elegans* blocks, were discarded. Third, a "simple merge" algorithm was used to merge adjacent blocks that had conserved order in *C. elegans* and *C. briggsae*. Finally, a dynamic programming algorithm was used to find the longest series of blocks having conserved order in *C. elegans* and *C. briggsae*. This algorithm bypassed short nonsyntenic blocks, enabling the creation of longer syntenic blocks. For each *C. briggsae* supercontig, the algorithm first found the longest series of contiguous blocks and merged these, and then found the next longest series using the blocks left over from the first iteration. This continued until no blocks remained. During this process, the merging of blocks that had conserved order was restricted such that the resultant merged blocks had to have similar sizes in the two species. This was done by restricting nonsyntenic gaps to less than 100 kb, and by not allowing gaps that would cause a greater than fivefold expansion of a syntenic block in either genome.

After careful merging of nucleotide-level alignments into syntenic blocks, it may be necessary to discard very small blocks. After merging *C. briggsae-C. elegans* WABA alignments into syntenic blocks, there was a large spike in the distribution of block size at approx 1250 bp. Many blocks of this size contained a single nucleotide-level alignment that correlated poorly with the positions of known orthologous genes. We considered these blocks to be unreliable, and so excluded all blocks of less than 1850 bp from the final analysis. This reduced the syntenic coverage of the genome by only 1.5%, but excluded 64% of merged blocks made by the "simple merge" step.

The true extent of synteny is underestimated when unfinished genomes are compared. This is because the ends of contigs are necessarily considered to be synteny breakpoints—when they may in fact be part of a contiguous syntenic
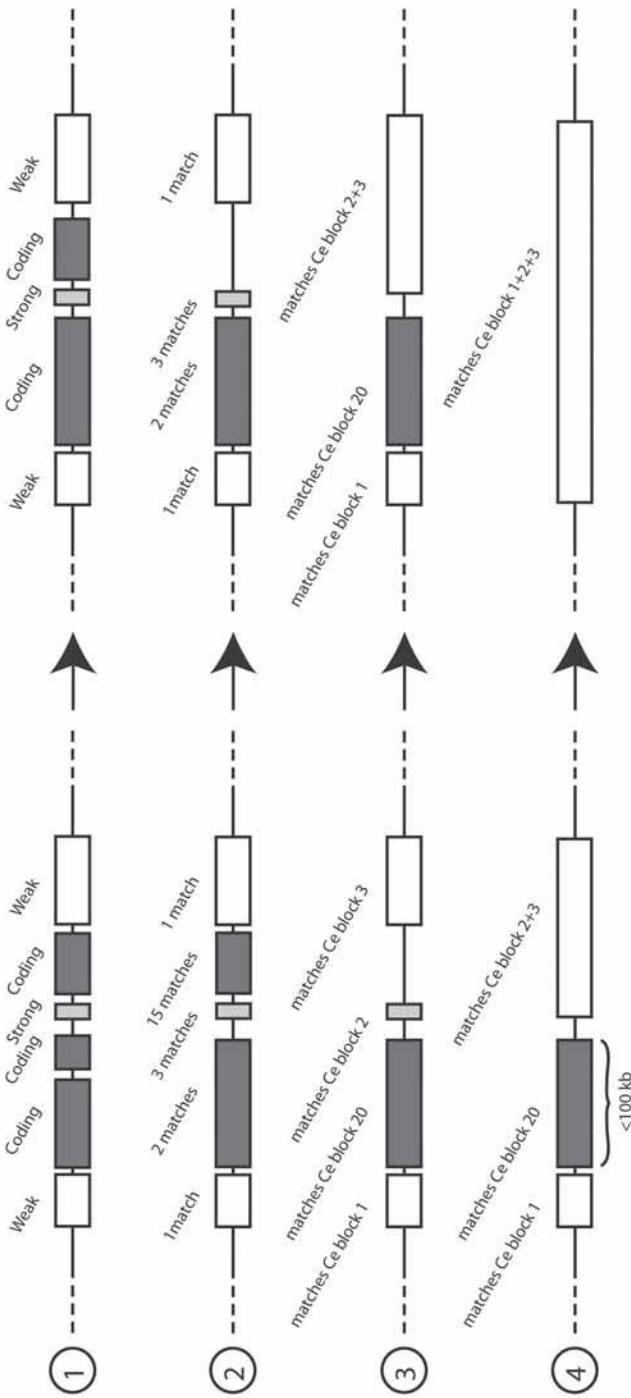
Fig. 3. The approach used by Stein et al. ([2](#)) to derive *Caenorhabditis elegans–Caenorhabditis briggsae* syntenic blocks from genome-wide nucleotide-level alignments. First, adjacent alignments that had the same WABA status ("weak," "strong," or "coding") were merged. Second, blocks that matched more than five blocks in the other species were discarded. Third, a "simple merge" algorithm was used to merge adjacent blocks that had conserved order in *C. elegans* and *C. briggsae*. Finally, a dynamic programming algorithm was used to find the longest series of blocks having conserved order in *C. elegans* and *C. briggsae*. This algorithm bypassed short nonsyntenic blocks of less than 100 kb, enabling the creation of longer syntenic blocks.

block. Comparisons to multiple species can resolve these ambiguities, because a contiguous breakpoint will rarely occur in the same relative position in several species' genome assemblies.

Analysis of synteny does not require whole genome nucleotide-level alignments. Synteny can be analyzed using any set of anchor points between two genomes, such as the positions of orthologous genes. As part of the *C. briggsae–C. elegans* analysis, a companion method to the alignment-based synteny analysis was developed, which used a conservative set of orthologous gene assignments *(2)*. This algorithm approaches the sensitivity of synteny analysis based on nucleotide-level alignments—but is faster and easier to implement. Orthologous genes (or other anchor features) are numbered in order along the contigs or chromosomes. Syntenic blocks are then defined by a series of orthologous genes that have conserved order between the two genomes. This approach overlooks syntenic regions that extend beyond gene boundaries, such as upstream regulatory sequences at the end of a syntenic block.

Newly sequenced (and less studied genomes) often lack contig-to-chromosome mappings. When comparing such a genome assembly to a finished genome that does have chromosome mappings, one can analyze synteny by numbering anchor points (nucleotide-level alignments or genes) in the newly sequenced genome according to the position of their orthologs in the second (finished) genome. This allows each contig of the newly sequenced genome to be placed and oriented on the second genome, according to the longest series of anchor points that have conserved order within that contig. This strategy works well for genomes with many anchor points and a large average contig size, but accuracy drops dramatically as the average contig size decreases.

5.3. Characterizing Breaks in Colinearity

With a set of merged non-overlapping syntenic blocks, it is relatively straightforward to classify breaks in colinearity. Each end of a syntenic block represents a potential break in synteny. By examining the neighboring blocks of each segment in relation to a reference genome, each block can be classified as an inversion, a transposition, or a reciprocal translocation *(2)*. Given an arrangement of syntenic blocks in *C. briggsae*:

=========== **a/b**————————————**c/d** ============

then the block **bc** was classified as an inversion if **a** was adjacent to **c** in *C. elegans*, and **b** was adjacent to **d** in *C. elegans*. On the other hand, the *C. briggsae* block **bc** was classified as a transposition if **a** and **d** were adjacent in *C. elegans*, and a reciprocal event could not be identified in *C. elegans*. Alter-

natively, the block **bc** was classified as involving one or two reciprocal translocations if another *C. briggsae* breakpoint was found:

============= **e/f** ——————

and **a** was adjacent to **f** in *C. elegans*, or **e** was adjacent to **d** in *C. elegans*.

5.4. Visualizing Syntenic Blocks

WormBase (http://www.wormbase.org *[4]*) provides several options for viewing syntenic blocks between *C. elegans* and *C. briggsae* graphically. First, in the Genome Browser, syntenic blocks are displayed under the "briggsae alignments" track. Within the syntenic blocks, WABA-classified "coding" regions appear in dark blue, "strong" regions in light blue, and "weak" regions in gray. Because syntenic blocks can contain gaps for which there is no WABA nucleotide-level alignment, they may not be fully continuous. A dashed gray line represents these gaps in a syntenic block. Syntenic blocks can be viewed from either the perspective of the *C. elegans* or the *C. briggsae* genome. Clicking on a syntenic block takes the user to the Synteny Browser. The Synteny Browser displays the relationship of syntenic blocks to both the *C. elegans* and *C. briggsae* genomes simultaneously.

6. Conclusion

The initial pairwise comparisons between *C. elegans* and *C. briggsae* built a foundation for the steps necessary for whole genome analyses of nematode genomes. We will soon have a data set of five different *Caenorhabditis* genomes. Comparison of these five genomes will both require and stimulate the development of novel techniques that make use of multispecies comparisons to predict genes and their regulatory sequences, to define orthologs and gene families, and to identify regions of chromosomal synteny. These analyses will provide the opportunity to study the evolution of nematode genomes in unprecedented detail.

Acknowledgments

References

1. Kiontke, K. and Sudhaus, W. (2006) Ecology of *Caenorhabditis* species, WormBook, ed. The *C. elegans* Research Community WormBook, doi/10.1895/ wormbook.1.37.1. Available at http://www.wormbook.org.

1a. The *C. elegans* Sequencing Consortium (1998) Genome sequence of the nematode *C. elegans*: a platform for investigating biology. *Science* **282,** 2012–2018.

2. Stein, L. D., Bao, Z., Blasiar, D., et al. (2003) The genome sequence of *Caenorhabditis briggsae*: a platform for comparative genomics. *PLoS Biol.* **1,** E45.

3. Sternberg, P. W., Waterston, R. H., Speith, J., Eddy, S. R., and Wilson, R. K. (2003) Genome sequence of additional *Caenorhabditis* species: enhancing the utility of *C. elegans* as a model organism. National Human Genome Research Institute. http://www.genome.gov/Pages/Research/Sequencing/SeqProposals/CaenorhabditisSEQ.pdf Accessed: April 2006.

4. Chen, N., Harris, T. W., Antoshechkin, I., et al. (2005) WormBase: a comprehensive data resource for *Caenorhabditis* biology and genomics. *Nucleic Acids Res.* **33,** D383–D389.

5. Worm ENCODE Consortium (2005) Worm ENCODE White Paper. http://www.wormbase.org/support_files/2005/worm_encode_white_paper.txt. Accessed: April 2006.

6. Salamov, A. A. and Solovyev, V. V. (2000) *Ab initio* gene finding in *Drosophila* genomic DNA. *Genome Res.* **10,** 516–522.

7. Howe, K. L., Chothia, T., and Durbin, R. (2002) GAZE: a generic framework for the integration of gene-prediction data by dynamic programming. *Genome Res.* **12,** 1418–1427.

8. Wei, C., Lamesch, P., Arumugam, M., et al. (2005) Closing in on the *C. elegans* ORFeome by cloning TWINSCAN predictions. *Genome Res.* **15,** 577–582.

9. Brent, M. R. and Guigó, R. (2004) Recent advances in gene structure prediction. *Curr. Opin. Struct. Biol.* **14,** 264–272.

10. Korf, I., Flicek, P., Duan, D., and Brent, M. R. (2001) Integrating genomic homology into gene structure prediction. *Bioinformatics* **17 Suppl 1,** S140–S148.

11. Siepel, A. C. and Haussler, D. (2004) Computational identification of evolutionarily conserved exons. In: *RECOMB 2004: Proceedings of the Eighth Annual International Conference on Molecular Biology*, March 27–31, 2004, San Diego, CA, ACM Press, New York, NY, pp. 177–186.

12. Pedersen, J. S. and Hein, J. (2003) Gene finding with a hidden Markov model of genome structure and evolution. *Bioinformatics* **19,** 219–227.

13. Liu, J., Tzou, P., Hill, R. J., and Sternberg, P. W. (1999) Structural requirements for the tissue-specific and tissue-general functions of the *Caenorhabditis elegans* epidermal growth factor LIN-3. *Genetics* **153,** 1257–1269.

14. Cho, S., Jin, S. W., Cohen, A., and Ellis, R. E. (2004) A phylogeny of *Caenorhabditis* reveals frequent loss of introns during nematode evolution. *Genome Res.* **14,** 1207–1220.

15. Wistrand, M. and Sonnhammer, E. L. (2005) Improved profile HMM performance by assessment of critical algorithmic features in SAM and HMMER. *BMC Bioinformatics* **6,** 99.

16. Long, M. (2001) Evolution of novel genes. *Curr. Opin. Genet. Dev.* **11,** 673–680.

17. Domazet-Loso, T. and Tautz, D. (2003) An evolutionary analysis of orphan genes in *Drosophila. Genome Res.* **13,** 2213–2219.

18. Coghlan, A. and Wolfe, K. H. (2004) Origins of recently gained introns in *Caenor-habditis*. *Proc. Natl. Acad. Sci. USA* **101,** 11,362–11,367.

19. Kiontke, K., Gavin, N. P., Raynes, Y., Roehrig, C., Piano, F., and Fitch, D. H. (2004) *Caenorhabditis* phylogeny predicts convergence of hermaphroditism and extensive intron loss. *Proc. Natl. Acad. Sci. USA* **101,** 9003–9008.

20. Llopart, A., Comeron, J. M., Brunet, F.G., Lachaise, D., and Long, M. (2002) Intron presence-absence polymorphism in *Drosophila* driven by positive Darwinian selection. *Proc. Natl. Acad. Sci. USA* **99,** 8121–8126.

21. Altschul, S. F., Madden, T. L., Schäffer, A. A., et al. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25,** 3389–3402.

22. Remm, M., Storm, C. E., and Sonnhammer, E. L. (2001) Automatic clustering of orthologs and in-paralogs from pairwise species comparisons. *J. Mol. Biol.* **314,** 1041–1052.

23. Li, L., Stoeckert, C. J., Jr., and Roos, D. S. (2003) OrthoMCL: identification of ortholog groups for eukaryotic genomes. *Genome Res.* **13,** 2178–2189.

24. van Dongen, S. (2000) Ph.D. thesis. *Graph clustering by flow simulation.* Dutch National Research Institute for Mathematics and Computer Science, University of Utrecht.

25. Eisen, J. A. (1998) Phylogenomics: improving functional predictions for uncharacterized genes by evolutionary analysis. *Genome Res.* **8,** 163–167.

26. Ohno, S. (1970) *Evolution by Gene Duplication.* Springer Verlag, NY.

27. Lespinet, O., Wolf, Y. I., Koonin, E. V., and Aravind, L. (2002) The role of lineage-specific gene family expansion in the evolution of eukaryotes. *Genome Res.* **12,** 1048–1059.

28. Chen, N., Pai, S., Zhao, Z., et al. (2005) Identification of a nematode chemosensory gene family. *Proc. Natl. Acad. Sci. USA* **102,** 146–151.

29. Enright, A. J., Van Dongen, S., and Ouzounis, C. A. (2002) An efficient algorithm for large-scale detection of protein families. *Nucleic Acids Res.* **30,** 1575–1584.

30. Ghedin, E., Wang, S., Foster, J. M., and Slatko, B. E. (2004) First sequenced genome of a parasitic nematode. *Trends Parasitol.* **20,** 151–153.

31. Webb, C. T., Shabalina, S. A., Ogurtsov, A. Y., and Kondrashov, A. S. (2002) Analysis of similarity within 142 pairs of orthologous intergenic regions of *Caenorhabditis elegans* and *Caenorhabditis briggsae. Nucleic Acids Res.* **30,** 1233–1239.

32. Schwartz, S., Kent, W. J., Smit, A., et al. (2003) Human-mouse alignments with BLASTZ. *Genome Res.* **13,** 103–107.

33. Batzoglou, S., Pachter, L., Mesirov, J. P., Berger, B., and Lander, E. S. (2000) Human and mouse gene structure: comparative analysis and application to exon prediction. *Genome Res.* **10,** 950–958.

34. Gaudet, J., Muttumu, S., Horner, M., and Mango, S. E. (2004) Whole-genome analysis of temporal gene expression during foregut development. *PLoS Biol.* **2,** E352.

35. Kirouac, M. and Sternberg, P. W. (2003) *cis*-Regulatory control of three cell fate-specific genes in vulval organogenesis of *Caenorhabditis elegans* and *C. briggsae.* *Dev. Biol.* **257,** 85–103.

36. Matys, V., Fricke, E., Geffers, R., et al. (2003) TRANSFAC: transcriptional regulation, from patterns to profiles. *Nucleic Acids Res.* **31,** 374–378.

37. GuhaThakurta, D., Schriefer, L. A., Waterston, R. H., and Stormo, G. D. (2004) Novel transcription regulatory elements in *Caenorhabditis elegans* muscle genes. *Genome Res.* **14,** 2457–2468.

38. Ao, W., Gaudet, J., Kent, W. J., Muttumu, S., and Mango, S.E. (2004) Environmentally induced foregut remodeling by PHA-4/FoxA and DAF-12/NHR. *Science* **305,** 1743–1746.

39. Tompa, M., Li, N., Bailey, T. L., et al. (2005) Assessing computational tools for the discovery of transcription factor binding sites. *Nat. Biotechnol.* **23,** 137–144.

40. Pavesi, G., Mereghetti, P., Mauri, G., and Pesole, G. (2004) Weeder Web: discovery of transcription factor binding sites in a set of sequences from co-regulated genes. *Nucleic Acids Res.* **32,** W199–W203.

41. Wang, X., Greenberg, J. F., and Chamberlin, H. M. (2004) Evolution of regulatory elements producing a conserved gene expression pattern in *Caenorhabditis.* *Evol. Dev.* **6,** 237–245.

42. Kent, W. J. and Zahler, A. M. (2000) Conservation, regulation, synteny, and introns in a large-scale *C. briggsae–C. elegans* genomic alignment. *Genome Res.* **10,** 1115–1125.

43. Blanchette, M., Kent, W. J., Riemer, C., et al. (2004) Aligning multiple genomic sequences with the threaded blockset aligner. *Genome Res.* **14,** 708–715.

3

WormBase

Methods for Data Mining and Comparative Genomics

Todd W. Harris and Lincoln D. Stein

Summary

WormBase is a comprehensive repository for information on *Caenorhabditis elegans* and related nematodes. Although the primary web-based interface of WormBase (http://www.wormbase.org/) is familiar to most *C. elegans* researchers, WormBase also offers powerful data-mining features for addressing questions of comparative genomics, genome structure, and evolution. In this chapter, we focus on data mining at WormBase through the use of flexible web interfaces, custom queries, and scripts. The intended audience includes users wishing to query the database beyond the confines of the web interface or fetch data *en masse*. No knowledge of programming is necessary or assumed, although users with intermediate skills in the Perl scripting language will be able to utilize additional data-mining approaches.

Key Words: WormBase; *C. elegans;* AceDB; AcePerl; bioinformatics; data mining; comparative genomics.

1. Introduction

WormBase is a web-accessible database of *Caenorhabditis elegans* and related nematodes *(1–4)* that serves the *C. elegans* research community and makes advances in the field accessible to the broader biomedical community. Born from the pioneering work of the genome database system AceDB *(5)*, WormBase has grown into an international consortium of researchers exploring methods of data analysis, warehousing, and visualization within the context of a highly developed model system.

The WormBase Consortium is composed of researchers at four locations: the California Institute of Technology (Caltech); the Genome Sequencing Center, Washington University (WashU); the Wellcome Trust Sanger Institute (Sanger); and Cold Spring Harbor Laboratory (CSHL). The duties of curating, analyzing,

and presenting data are split between the groups. Caltech is primarily responsible for systematic curation of data from the published literature; WashU and Sanger are each responsible for curating half of the genome sequence (and associated gene models); CSHL is in charge of user-interface development and management of the website. Additionally, Sanger is responsible for assembling the release version of the database. This process merges the local databases and performs various analyses as necessary (e.g., BLAST analysis). The final database is distributed to CSHL where the data is made available to our users on the main website and official mirror sites.

WormBase contains a vast array of information mirroring the breadth and depth of the *C. elegans* research field. These data include the sequence and structure of the *C. elegans* and *Caenorhabditis briggsae* genomes *(6,7)*; genetic information such as strains, alleles, and genetic maps; data from large-scale experiments such as genome-wide RNAi screens *(8–11)* and the ORFeome project *(12,13)*; reagents such as antibodies, polymorphisms, and transgenes; gene and anatomy ontologies; curated functional descriptions; cellular data such as expression patterns and the complete lineage and connectivity maps *(14–16)*; and comparative data such as orthologs and syntenic regions between species. Over the next year, WormBase will add the complete genomic sequence and annotations of three additional nematodes (*Caenorhabditis remanei*, *Caenorhabditis japonica*, and *Caenorhabditis* n. sp. PB2801).

In addition to web-based views of the data at http://www.wormbase.org/, a number of methods for data mining and comparative genomics are available for exploring the wealth of information housed at WormBase. These tools facilitate custom views of the data and the retrieval of specific datasets for further analyses.

1.1. Access Options

The most commonly used point of entry to WormBase is via the web interface at http://www.wormbase.org/. Additionally, WormBase has built a number of mirror sites to better serve our user base across the globe. A full listing of mirrors is available in the appendix; a list of current mirror sites is always displayed on the front page of WormBase. WormBase also maintains a development server (http://dev.wormbase.org) where new features and datasets are tested before being made available on the primary site. Users are welcome to use this site but are cautioned that the interface and data may not be stable. Finally, WormBase offers a publicly accessible data-mining server (aceserver. cshl.org; discussed in **Subheading 3.4.**).

1.2. Release Cycle, Versioning, and Data Freezes

WormBase follows a regular release cycle, currently one release approximately every 3 wk. This ensures timely access to new datasets and curator-con-

firmed annotations. Releases are versioned using the format "WSXXX" (e.g., WS100, WS101, and so on), with the current version of WormBase always displayed on the home page. Each new WormBase release is generated anew from the latest set of source databases from each WormBase group.

With every 10th release of the database, we create a data and software "freeze" and make it available in perpetuity at http://[ws_version].wormbase.org/. These freezes serve two purposes. First, they ensure that the web interface for a particular freeze will continue to be available. This is important because the display layer of WormBase aggregates information in a way that is not readily available from the database itself. Furthermore, software development occurs in tandem with database releases and the two must be matched for consistent results. Second, freezes provide a stable referential release for ease of citation and verification. Collaborating groups can coordinate analyses by using the same version of the data. This is especially important when considering that the genome sequence undergoes small corrections periodically, potentially affecting any analysis dependent on the chromosomal location of features. Users conducting such studies are encouraged to use the most recent frozen version of WormBase.

1.3. Citing WormBase

How should users cite data obtained from WormBase? Direct links to the database can be cited as a full URL copied directly from a browser. Users should also make note of the particular release the data was obtained from (displayed on the home page). A sample citation might look like:

```
http://www.wormbase.org/db/gene/gene?name=unc-26;
WormBase Release WS140.
```

Visit http://www.wormbase.org/about/citing.html for the most up-to-date information on citing WormBase.

1.4. Informatics Infrastructure

WormBase is comprised of three components critical to effective data mining: two database systems and the software that powers the website. AceDB is the primary database system. Curation centers maintain local datasets in AceDB and AceDB delivers much of the information displayed on the website. To facilitate fast queries of genomic annotations, some data is extracted from AceDB and deposited in secondary relational databases. These databases are created with the Bio::DB::GFF data schema and housed in MySQL. The web interface is generated by a collection of Perl scripts that interact with both the AceDB and MySQL databases.

1.4.1. The WormBase (AceDB) Data Model

Effective data mining requires an understanding of the underlying AceDB data model. AceDB uses an intuitive object-oriented structure that is well suited

for biological data. An AceDB data model consists of a series of structured classes stored in a human readable format. Each class can have a number of tags, which can point to various data types or cross-references to other objects. The presence or absence of some tags carry specific information. For example, the Gene class (**Fig. 1**) defines tags such as "Corresponding_CDS" and "Variation" to store references to the appropriate CDS and variation objects for a given gene. The "Live" tag has no subtags; its presence (or absence) indicates whether the gene is current (or has been retired). Individual tags can be accessed by name from instantiated objects, sparing programmers the tedium of complicated table joins typical of relational database systems.

The easiest way to become familiar with the data model used at WormBase is through the web interface. The contextual navigation bar on every data-bearing page on the website contains two links for exploring the data model. The "Schema" link displays the data schema for the class of the current object. The "Tree Display" link shows the schema populated with data from the current object. One can search for the model of a specific class from the home page by typing in a class name and selecting "Model" from the popup menu. Questions about the model should be addressed to help@wormbase.org. Additional information on the structure of AceDB data models is available at http://www.acedb.org/.

1.4.2. Overview of Prominent Classes

The AceDB database at WormBase spans almost 100 classes (prominent classes are described in **Table 1**). Although class names usually reflect the type of objects they are meant to represent, this is not always transparent because of generalizations in the data model or historical constraints. For example, the Gene class includes a variety of biologically distinct concepts, such as genetic loci, noncoding RNAs, and predicted genes. An easy way to become familiar with the various classes at WormBase is through the Class Browser page at http://www.wormbase.org/db/searches/class_query.

1.4.3. Object Identifiers

All objects in WormBase drawn from the AceDB database have an associated *class* and *name*. To discover the class of a particular object, search for it from the main page using the "Anything" option. The class and name will be displayed in the URL. For most classes, object names correlate with the most common name of the object itself. Recently, however, WormBase has moved towards serialized identifiers for a number of classes. This makes data management and versioning substantially easier and allows for distinct objects to have the same public name. These classes typically have a *_name companion class that serves to map publicly used names to the serialized object name. Classes using serialized identifiers include Gene, RNAi, Paper, and Variation.

```
?Gene    Evidence #Evidence
 SMap S_parent UNIQUE Sequence UNIQUE ?Sequence XREF Gene_child
 Identity Version UNIQUE Int
          Name   CGC_name        UNIQUE ?Gene_name XREF CGC_name_for #Evidence
                 Sequence_nameUNIQUE ?Gene_name XREF Sequence_name_for
              Molecular_name       ?Gene_name XREF Molecular_name_for
                 Other_name          ?Gene_name XREF Other_name_for #Evidence
                 Public_name     UNIQUE ?Gene_name XREF Public_name_for
                 Species UNIQUE ?Species
                 Live
 Gene_info Gene_class UNIQUE ?Gene_class XREF Genes
          Laboratory ?Laboratory
          Reference_allele ?Variation Text
          Allele ?Variation XREF Gene #Evidence
          Phenotype ?Text #Evidence
          Strain ?Strain XREF Gene
          GO_term ?GO_term XREF Gene ?GO_code #Evidence
          Contained_in_operon ?Operon XREF Contains_gene
          Ortholog ?Gene XREF Ortholog ?Species #Evidence
          Structured_description Provisional_description ?Text
                                 Detailed_description ?Text #Evidence
                                 Concise_description ?Text #Evidence
                                 Other_description ?Text #Evidence
                                 Biological_process ?Text #Evidence
                                 Molecular_function ?Text #Evidence
                                 Expression ?Text #Evidence
 Molecular_info Corresponding_CDS ?CDS XREF Gene #Evidence
 Experimental_info RNAi_result ?RNAi XREF Gene
                 Expr_pattern ?Expr_pattern XREF Gene
                 Drives_Transgene ?Transgene XREF Driven_by_gene
                 Transgene_product ?Transgene XREF Gene
                 Rescued_by_transgene ?Transgene
                 Gene_regulation Trans_regulator ?Gene_regulation
                 Antibody ?Antibody XREF Gene
                 Microarray_results ?Microarray_results XREF Gene
 Map_info  Map UNIQUE ?Map XREF Gene #Map_position
          Hide_under ?Gene XREF Representative_for
          Representative_for ?Gene XREF Hide_under
          Negative_clone ?Clone XREF Negative_gene #Evidence
          Mapping_data  2_point ?2_point_data
                        Multi_point ?Multi_pt_data
                        Pos_neg_data ?Pos_neg_data
 Reference ?Paper XREF Gene
 Remark ?Text #Evidence
 Method UNIQUE ?Method
```

Fig. 1. A partial listing of the AceDB Gene class. Objects referenced in the model are preceded by a "?." Tags may store multiple items to their right, including text and object cross-references. Cross-references (denoted by "XREF") serve to associate specific tags in related objects to the current object. Note that some tags accept no data to their right (e.g., "Live"). These tags carry information according to their presence or absence. Another important element used throughout the data model is the evidence hash (denoted using the nomenclature "#Evidence"). Evidence hashes store information pertaining to the source of individual pieces of data.

Table 1
Prominent Classes in the AceDB Data Model at WormBase

Object	Name	Class	Description/notes
A predicted gene	A cosmid dot name, such as JC8.10a	Gene_name	The Gene_name class serves to relate commonly used gene names to the serialized names used for Gene objects.
A genetic locus	A CGC standard locus name, such as lin-29	Gene_name	Like predicted genes, the Gene_name class relates publicly used names to the Gene class
A gene	A serialized WBGeneID	Gene	Gene objects are the top-level containers for genetic loci, molecularly identified genes, and RNAs.
A Genbank Accession Number (protein or nucleotide)	The accession number	Accession_Number	This will retrieve an Accession_Number object which is a list of WormBase names that correspond to that accession number.
A protein	A wormpep accession number, proceeded by "WP:", as in WP:CE28571	Protein	The corresponding Gene object of a protein can be fetched by searching the Gene_name class.
A clone	The cosmid or YAC name, for example T20H4	Clone	
A cell	A cell name, such as M1	Cell	
An RNAi experiment	A WBRNAi identifier	RNAi	Like the Gene class, WormBase uses serialized identifiers for the RNAi class. Previously used experiment identifiers (such as JA:59E12.2) are retained under the History_name tag.
An allele or polymorphism	An allele name, such as e345 or a WBVariation identifier	Variation	The Variation class uses serialized identifiers.
Genomic sequence	The cosmid or YAC from which the sequence was obtained, for example Y67H2	Genomic_Sequence	A generic class name of sequence is also recognized

2. Materials

Data mining at WormBase requires only modest computational power. Any current personal computer will be suitable for web-based data-mining approaches. Users wishing to access the resource programmatically using the methods described next will need access to an operating system that supports the Perl programming language.

3. Methods

WormBase offers four data-mining options to meet the needs of our users. In increasing order of complexity and flexibility are web-based data mining pages, query languages, programmatic mining of the web interface, and scriptable access via the AcePerl and Bio::DB::GFF programming interfaces. In the listings that follow, query and code examples are displayed in `a fixed width font`. The WormBase data-mining archive (http://www.wormbase.org/data_mining/) contains additional sample queries and scripts as well as expanded versions of the scripts described beginning in **Subheading 3.2.1**.

3.1. Web-Based Data-Mining Options

WormBase offers multiple web-based data-mining options. WormMart (the WormBase implementation of BioMart *(17)*; http://www.wormbase.org/Bio Mart/martview) is the newest addition to our data-mining repertoire. WormMart provides a flexible, user-friendly interface for retrieving select data from WormBase *en masse*. Users begin by selecting a reference release of the database followed by a point of focus (e.g., a gene). Results can be restricted to a list of identifiers or chromosomal or genetic map positions. Next, a series of filters can be enabled further restricting the results returned. For example, a user might choose to include only those genes that have alleles. Finally, a broad range of data can be selected that should be included in the output.

The "Batch" scripts (Batch genes and Batch sequences) offer access to annotations and sequences, respectively. Both pages support searching with a list of gene names or identifiers, the use of wildcards, and HTML or plain text output. It is anticipated that WormMart will eventually replace the "Batch" scripts. WormBase also provides a number of tools for querying specific datasets, such as RNAi results, expression patterns, and microarray data. These tools are described in more detail on the WormBase site map (http://www.wormbase. org/db/misc/site_map).

3.2. Query Languages

WormBase offers two query languages, both of which were developed as part of the underlying AceDB database system. Queries written in these languages can be submitted to the database through web forms. Because query

languages work on the database directly, users can retrieve data that may not be displayed on the website, in a form amenable to further processing. Successful queries require familiarity with the underlying data model.

3.2.1. WormBase Query Language

Of the two query languages offered at WormBase, the WormBase query language (WQL) is the easier to use. In general, WQL queries take the structure of "find CLASS [PROPERTIES]" where CLASS is the class of interest and PROPERTIES are filters to apply to the returned keyset. WQL queries support the use of logical operators (NOT, OR, AND, and so on) and wildcard characters (? and *). The following examples can be submitted at http://www.wormbase.org/db/searches/wb_query. This page can also be found from either the "Site Map" or "Searches" link on the primary navigation bar at the top of every page.
Fetch all *C. elegans* genes in the database.

```
find Gene WHERE Species="Caenorhabditis elegans"
```

Find a list of genes that might encode actin proteins.

```
find Gene WHERE Public_name="act-*"
```

Fetch all genes that are cloned and have alleles. Here we test for the existence of the "Allele" and "Corresponding_CDS" tags in Gene objects. This will return genetically defined and cloned genes, as well as those identified through reverse genetics.

```
find Gene WHERE Allele AND Corresponding_CDS
```

To connect individual queries together, use a semicolon. The second query will be run against the results of the first query. The following example will find all CDSs that are confirmed by the presence of a cDNA.

```
find CDS WHERE Prediction_status="Confirmed"; Matching_cDNA
```

To traverse from one class, to another, use the "Follow" command. The following command will fetch all genes that have an Emb RNAi phenotype on Chromosome III. In this query, we first find all RNAi experiments that result in an Emb phenotype, then "Follow" these results to a corresponding set of Gene objects. Finally, we select a subset of the initial results based on chromosomal location.

```
find RNAi WHERE Phenotype="Emb"; Follow Gene; Map="III"
```

When querying subtags, the primary tag must be specified and the subtag preceded by a "#." The "HERE" clause lets an additional condition be applied to the same tag. It is important to note that the query operators AND, OR, NOT, and HERE must be in all capitals; the remainder of the query can be in lowercase. This query will find all Variation objects between two genetic map points, and then selects a subset of polymorphisms based on the presence of the "SNP" tag.

```
find Variation Map="I" # (Position >= 5.0 AND HERE <= 10.0) AND (SNP)
```

3.2.2. AceDB Query Language

The AceDB query language uses an expanded syntax that facilitates more complicated queries. In particular, AceDB query language offers a structured query language-style syntax that lets users select the columns (in this case tags) to include in the output from a given key set. The following example queries against the database can be submitted at http://www.wormbase.org/db/searches/aql_query.

Fetch all genes in the database. Here we use an alias (a) to represent instances of Gene objects fetched by the query.

```
select a from a in class Gene
```

Find all genes in the database, including their WormBase WBGeneID and their more commonly used public name.

```
select a,a->Public_name from a in class Gene
```

Find all alleles that have phenotype data but lack sequence information. This example checks for the presence and absence of the "Phenotype" and "Sequence" tags, respectively, returning a list of variations.

```
select all class Variation where exists_tag->Phenotype and
not exists_tag->Sequence
```

Fetch all polymorphisms that occur within genes, reporting the polymorphism name and containing gene. This query checks for the presence of the "SNP" and "Gene" tags of Variation objects in a boolean context.

```
select a,a->Gene from a in class Variation where
exists a->SNP[0] and exists a->Gene[0]
```

3.3. Programmatically Mining the Web Interface

A first step toward programmatic data mining at WormBase is to create scripts that extract data directly from pages on the website. This approach is often referred to as screen-scraping. Screen-scraping scripts can be created with limited knowledge of a programming language such as Perl. Although not an official WormBase data mining method, the ease with which these scripts can be created make them a viable strategy for fetching data from the site.

Screen-scraping offers several advantages over more direct data-mining approaches. Users need not be familiar with the data model in order to write effective scripts. Furthermore, the web displays at WormBase often integrate data from several different classes in a single comprehensive display. Often it is simpler to screen-scrape this data rather than trying to recreate the logic of a script using programmatic interfaces to the database. Because screen-scraping relies on parsing the HTML of a page, scripts are susceptible to changes in the document. Also, because they are limited to data presented on the visual interface, they are not as flexible as approaches that mine the data-

base directly. Nevertheless, screen-scraping can be a quick way to access a collection of data presented on the website.

The following script takes a list of loci (hard-coded in the script itself) and fetches the "Concise Descriptions" displayed on the "Gene" page (http://www. wormbase.org/db/gene/gene). *See* **Notes 1** and **2** for information on running this script on your system.

```
1   #!/usr/bin/perl -w
2   # This script demonstrates one way to "screen scrape" WormBase
3
4   use strict;
5   use WWW::Mechanize;
6
7 # The URL of the target page
8 use constant URL =>
9          'http://aceserver.cshl.org/db/gene/gene?name=';
10
11 # A list of genes to fetch
12 my @genes = qw/unc-2 unc-26 unc-70 unc-119 dyn-1 vab-3/;
13
14 foreach my $gene (@genes) {
15     my $agent = WWW::Mechanize->new();
16     $agent->get(URL . $gene); # Create the full URL
17     $agent->success
18      or die "Sorry, I couldn't fetch the page for $gene: $!";
19     my $content = $agent->content;
20
21     # Parse out the Concise Description.
22     my ($description) =
23      ($content =~ /Concise\sDescription.*?body\">(.*?)\s\[/);
24
25     print "$gene\t$description\n";
26     sleep 3;
27 }
```

This script uses standard Perl notation (*see* **Note 3**), executing the following steps. Line 5 makes functions provided by the WWW::Mechanize library available to the script. In lines 8–9, a constant is defined containing a fragment URL of the page to mine for information. In line 12, we specify a list of genes to fetch concise description for. Line 14 begins a loop iterating over each of the values in the `@genes` array. Line 15 creates a WWW::Mechanize object which will handle interactions with the website. We store the object in the `$agent` variable. In line 16, we use the WWW::Mechanize agent to fetch the page for the current gene by concatenating the URL constant value to the current value of `$gene`. Lines 17–18 verify that the request was successful using the `success()` method of WWW::Mechanize in boolean context. If the request was succesful, line 19 fetches the content of the page, storing it in the `$content` variable. Note that `$content` contains HTML formatting, not just the text visible on the web page. Lines 22 and 23 are the most critical elements of this script. This regular expression parses out the text that comes immediately after "Concise Description" on

the web page, storing it in the `$description` variable. Line 25 prints out the gene name and description parsed from the page. Lines 26 and 27 are the end of the loop, with a 3-s delay before the next iteration.

Out of courtesy to other users, we request that users screen-scraping Worm-Base direct their scripts to http://aceserver.cshl.org/ and that they include a 3-s delay between requests. Our full acceptable use policy is available at http://www.wormbase.org/about/acceptable_use.html.

3.4. Programmatic Access to AceDB Using AcePerl

Although screen-scraping approaches are fast and easy to implement, they are limited to data that is visible on the web interface. The AcePerl Perl module (*19*) circumvents these limitations by providing direct access to the AceDB database at WormBase. AcePerl is particularly useful for data-mining tasks seeking text-based annotations or those that need to cross several different database classes. Using AcePerl requires beginner to intermediate knowledge of Perl including a familiarity with the Perl object-oriented idiom.

WormBase offers a publicly accessible data-mining server open to scripts using AcePerl. To use this server, scripts should be directed to aceserver.cshl.org, port 2005. Alternatively, queries can be directed against a local data source. The following script demonstrates how to fetch all known alleles for all *C. elegans* genes from *aceserver* (*see* **Note 4** for information on the WormBase representation of genes). In order to develop AcePerl scripts or test the example script, the AcePerl module must be installed (*see* **Note 5**).

```
1   #!/usr/bin/perl -w
2   use strict;
3   use Ace;
4
5 my $DB = Ace->connect(-host => 'aceserver.cshl.org',
6                       -port => '2005')
7            or die "Couldn't connect to aceserver: $!";
8
9 my @genes = $DB->fetch(-query=>
10     qq{find Gene where Species="Caenorhabditis elegans"});
11
12 foreach my $gene (@genes) {
13   my $name = $gene->Public_name || $gene;
14   my @alleles = $gene->Allele;
15   print join("\t",$name,@alleles),"\n";
16 }
```

This script uses standard Perl notation (*see* **Note 3**), executing the following steps. Line 3 makes functions provided by the AcePerl library available to the script. Lines 5–7 establish a connection to the appropriate database, exiting the script if a connection cannot be established. In lines 9–10, a query is executed on the AceDB database, fetching all *C. elegans* genes. *See* **Note 6** for an example of fetching genes by their public name. Line 12 begins a loop iterating over

all genes returned by the query. In line 13, we set the name of the gene to the "Public_name" tag of the object (if it exists) or else it becomes the name of the object itself. Line 14 fetches all known alleles for the gene and stores them in the `@alleles` array. Line 15 prints the name of the gene and a list of its alleles to standard output and the loop is ended.

3.5. Programmatic Access to GFF Databases Using Bio::DB::GFF

To retrieve genomic annotations (including anything that may have sequence coordinates), use the Bio::DB::GFF Perl API (part of the BioPerl project *[19]*). The Bio::DB::GFF module provides methods for creating and querying relational databases of genomic annotations. These databases are created from tab-delimited files of genomic annotations in the GFF format. Files contain one annotation per line with fields describing its reference sequence (typically a chromosome), its source (how the annotation was derived), its method (the type of annotation), and various other attributes such as strand, phase, and start and stop coordinates. WormBase currently uses two Bio::DB::GFF databases, one each for *C. elegans* and *C. briggsae* genomic annotations. The BioPerl suite of modules must be installed (available from CPAN or www.bioperl.org) in order to develop scripts utilizing Bio::DB::GFF databases.

Features may be fetched from Bio::DB::GFF databases using feature names, coordinates, ranges, or the source and method of the annotation, usually presented as a method:source couplet. For example, to fetch the coordinates of all CDSs in the database, one might query for "curated:CDS." The GFF methods and sources currently in use at WormBase are displayed at http://www.worm base.org/db/misc/database_stats. Grouped features (like the introns and exons of a transcript; *see* **Note 7**) can be aggregated together during retrieval. Features can be reported in relative or absolute coordinates (and converted between the two as desired).

The following example script can generically fetch any sequence feature from the *C. elegans* GFF database on the WormBase data-mining server (aceser ver.cshl.org), optionally fetching the DNA of the feature in FASTA format (*see* **Notes 8** and **9** for information on how to run this script).

```perl
1 #!/usr/bin/perl -w
2
3 use strict;
4 use Bio::DB::GFF;
5
6 my $feature = shift;
7 my $dna = shift;
8 $feature || die "You must specify a feature to fetch…\n";
9
10 # Establish a connection to the appropriate data source
11 my $db = Bio::DB::GFF->new(-dsn =>
12             'dbi:mysql:elegans:aceserver.cshl.org',
```

```
13                            -user => 'anonymous')
14              || die "Couldn't establish a connection to DSN: $!";
15
16 # Fetch an iterator of the requested feature
17 my $iterator = $db->get_seq_stream(-type => $feature);
18 while (my $feature = $iterator->next_seq) {
19
20   # Create an informative header
21   my $name = $feature->name;
22   my $type = $feature->type;
23   my ($start,$stop) = ($feature->start,$feature->stop);
24   my $refseq = $feature->sourceseq;
25   my $header = "$name ($type; $refseq:$start..$stop)";
26
27 # If requested, fetch sequence of the feature in FASTA
28 if ($dna) {
29   my $seq = to_fasta($feature->dna);
30   print ">$header\n",$seq,"\n";
31 } else {
32   print "$header\n";
33 }
34 }
35
36 # This subroutine converts a dna string into fasta format
37 sub to_fasta {
38   my $sequence = shift;
39   return if ($sequence=~/^>(.+)$/m); # Return if already in FASTA
40   $sequence =~ s/(.{80})/$1\n/g; # Carriage return
41  return $sequence;
42 }
```

This script uses standard Perl notation (*see* **Note 3**), executing the following steps. Line 4 makes the Bio::DB::GFF library available to the script. Lines 6–8 accept input form the command line: the required feature to fetch, and an optional flag to fetch DNA if desired. In lines 11–14, the script attempts to connect to the GFF database on aceserver.cshl.org using the `connect()` method of Bio::DB::GFF, exiting if a connection cannot be established. Line 16 fetches a feature stream from the database of the desired feature. In line 18, a loop is begun that iterates over all features of the specified type. In lines 21–24, a variety of information about the current feature is fetched and stored in variables; these are used to create a FASTA style header in line 25. Lines 28–34 generate the primary output of the script. If the "dna" option is specified on the command line, the sequence of the feature is fetched in FASTA format and printed along with the header in lines 29 and 30; if not, the header line itself is printed in line 32. The primary loop ends in line 34. Lines 37–42 comprise a subroutine that converts a sequence into FASTA format.

3.6. Running WormBase Locally

For users requiring high speed—such as those conducting large amounts of data-mining experiments—a local installation of WormBase is an attractive

option. Local installations are not limited by server or network congestion or even the need for Internet access. An installation can be limited to the AceDB and GFF databases in use at WormBase, or can include the software that drives the WormBase site as well. Local installations of WormBase also benefit from the ability to use AceDB directly (including the graphical and text-based interfaces to AceDB databases, *xace* and *tace*).

Hardware requirements differ based on the components of WormBase you choose to install and how the site will be used. Minimally, a 1-Ghz G4 or 900 Mhz Pentium III processor are required for acceptable performance, running either Mac OS X or a Unix/Linux variant. WormBase can run acceptably for a small number of users with 1 GB of memory, but a minimum of 4 GB memory is recommended for server installations. The AceDB and GFF databases currently require about 10 and 4 GB of disk space, respectively. Users should expect disk space requirements to grow steadily with the scheduled addition of three new genomes. These relatively modest requirements mean that a desktop machine or laptop can be suitable for running a local copy of WormBase. For installations serving multiple users, a server class machine should be considered. We refer users to the current documentation describing the installation procedure at http://www.wormbase.org/docs/INSTALL.html.

For convenience, we provide prepackaged files of the database components of WormBase for every release. Currently, this consists of four files: the AceDB database, the *C. elegans* GFF database, the *C. briggsae* GFF database, and databases to support the BLAST and BLAT searches. These packages greatly simplify local installations. The current versions of these files, as well as documentation on how to install them can be found at ftp://ftp.wormbase.org/pub/wormbase/mirror/database_tarballs/.

The software that drives WormBase can be obtained through several avenues. The recommended method is to retrieve the software by anonymous rsync access. This ensures direct synchronization to the current WormBase site. Alternatively, compressed archives of the software corresponding to each data release are available on the WormBase FTP site. Finally, we offer anonymous CVS access to the WormBase code (*see* http://www.wormbase.org/docs/INSTALL.html for additional information). The Bio::GMOD Perl module (available on CPAN) contains a number of scripts for automating updates of a WormBase installation.

3.7. Linking to WormBase

To generate a link to an object in WormBase, specify the class and name of the object using a URL with the following structure:

```
http://www.wormbase.org/db/get?name=WBGene00006763;class=gene
```

You can also fetch an XML representation of an object by linking to the following URL:

```
http://www.wormbase.org/db/misc/xml?name=WBGene00006763;class=Gene
```

And similarly for a text-only display of the object:

```
http://www.wormbase.org/db/misc/text?name=WBGene00006763;class=Gene
```

To link to an image of the genome, use a URL of the following format:

```
http://www.wormbase.org/db/seq/gbrowse/wormbase?name=unc-26
```

The "name" parameter can accept almost any item that might have coordinates on the physical map, or a physical span (e.g., `III:30000..50000`). *See* http://www.wormbase.org/data_mining/linking.html for the most current linking rules.

3.8. Displaying Remote Annotations on the Genome Browser

An often overlooked but very useful feature of the Genome Browser is the ability to display remotely hosted annotations within the context of annotations housed at WormBase *(20)*. Annotations can be entered as plain text through a web form or by uploading a file as described in the "Upload your own annotations" link at the bottom of the Genome Browser. Uploaded annotations are also included in images generated from the "Publication Quality Image" link. This option creates a scalable vector graphics image of the current genome span. Scalable vector graphics images can be edited in vector-based graphics applications, enlarged or compressed with no loss in quality, and submitted as high quality figures for publication.

To publish preliminary data sets or remote annotations, users may make use of the distributed annotation system (DAS) *(21)*. Using DAS, annotations are hosted on a remote server but displayed in the context of WormBase by a DAS client (in this case the Genome Browser). These annotations can be made public simply by notifying WormBase of the desire to make the data available. We can assist in creating a custom option that allows WormBase users to enable display of the remote data.

3.9. Tools for Comparative Genomics

WormBase provides several useful tools and precomputed datasets for comparative genomics analyses. First, WormBase houses the entire *C. elegans* and *C. briggsae* genomic sequences and predicted gene and protein sets. Second, precalculated reciprocal best mutual match BLASTP orthologs between *C. briggsae* and *C. elegans* are displayed on the Gene Summary page (when known). Finally, WormBase displays nucleotide level alignments between *C. elegans* and *C. briggsae* on both the Genome Browser and the Synteny Browser. We will extend the utility of WormBase for comparative genomics analyses in the coming year with the addition of three additional *Caenorhabditis* genomes.

Nucleotide level alignments identified through use of the WABA algorithm *(22)* are displayed on both the Genome Browser and the Synteny Browser. WABA distinguishes between "coding," "strong," and "weak" alignments. Prior to display, these alignments are post-processed to generate a series of syntenic blocks. On the Genome Browser, such merged syntenic blocks *(7)* are displayed under the "*Briggsae* alignments" track using the following color scheme to distinguish the strength of the alignment: dark blue (coding), light blue (strong), and gray (weak). Because merged blocks can contain gaps where there was no distinct nucleotide alignment, a single syntenic segment on the Genome Browser may not be continuous. Such gaps within a given syntenic block are represented by a dashed gray line. Clicking on one of these alignments will take the user to the Synteny Browser where syntenic blocks can be viewed in relation to both genomes simultanously.

4. Notes

1. To execute the Perl example scripts, a system with Perl v5.6.0 or greater is required. Scripts can be entered manually into a text editor or word processing program and saved to disk, or downloaded directly from http://www.wormbase.org/data_mining/ in order to avoid data entry errors. If typing in the example scripts manually, do not include the listed line numbers. After the script has been entered or downloaded it needs to be made executable by entering the following commands at a command line prompt.
   ```
   shell> chmod 770 example_script.pl
   shell> ./example_script.pl
   ```
   To capture the output of any script, redirect standard output to a filename using the following notation.
   ```
   shell> ./example_script.pl > output.txt
   ```

2. This example script uses the WWW::Mechanize module, available from CPAN. This can be installed from a command line prompt (denoted by "`shell>`" below), using the following commands.
   ```
   shell> sudo perl -MCPAN -e shell
   cpan> install WWW::Mechanize
   cpan> quit
   ```
   You will need superuser privileges on your system in order to install WWW:: Mechanize as indicated. Consult your system administrator if you do not have these privileges. *See* **Note 1** for how to execute the script.

3. The Perl programming language uses the following general conventions. Perl scripts typically begin with the notation "`#!/usr/bin/perl`" which indicates that the file is a Perl script and where the Perl binary is installed on your system. Lines in which the first character is a "#" are comments in the code and ignored by the Perl interpreter (with the exception of the first line). The "use strict" pragma, although not required, specifies that all variables must be explicitly declared. Lines in a Perl script can continue onto subsequent lines; a semi-colon indicates the end of a line. Perl uses three variable types common to most pro-

gramming languages: scalars, arrays, and associative arrays. Scalar variable names are proceeded with a "$" (e.g., **$variable**) and can contain either strings or numeric values. Arrays begin with an "@" (e.g., **@list**). Associative arrays or hashes contain key-value pairs and begin with a "%" (e.g., **%hash**). For additional information, consult a tutorial on Perl *(23)* or the built in Perl documentation on your system.

4. When implementing a data mining approach, it is important to consider how WormBase represents genes, CDSs, and transcripts in the data model. WormBase has implemented a unified Gene class intended as a top-level container for all data logically associated to a particular unit of a chromosome. Each Gene object may have zero, one or many associated CDSs listed under the "Corresponding_ CDS" tag, corresponding to alternative splices in the coding sequence. Genes lacking CDSs are usually uncloned genes, noncoding genes, or pseudogenes. Finally, each CDS object may have one or more associated transcript objects (stored under the "Corresponding_transcript") corresponding to alternative splicing in UTRs.

5. In order to execute this script, you will need to install AcePerl. This is done most easily via the Perl CPAN module. From a command line prompt, the following stanzas will install AcePerl on most 32-bit Unix-based systems.

```
shell> sudo perl –MCPAN –e shell
cpan> install Ace
// choose options 2 or 3, accept all other defaults
```

Once installed, you can read more about using AcePerl using the following command.

```
shell> perldoc Ace
```

6. To fetch Gene objects from AceDB using commonly used locus or molecular identifiers, first query the "Gene_name" class, then fetch the corresponding Gene object:

```
$gene_name = $DB->fetch(Gene_name => 'unc-70');
$gene = $gene_name->Public_name_for;
```

The "Gene_name" class provides a convenient mechanism for mapping the many publicly used names for a gene to a single gene object. Using the example above as a guide, one can search for gene objects using protein or locus names or molecular identifiers.

7. An understanding of how sequence features are represented in the GFF data schema is essential for data mining. In the current GFF databases, WormBase represents a single span corresponding to the maximal extents of a gene as Corresponding_transcript:Transcript (source:method). Similarly, a single full length span corresponding to just the CDS is listed as curated:CDS. The component parts of a full length transcript are Coding_transcript:intron, Coding_ transcript:coding_exon, Coding_transcript:three_prime_UTR and Coding_ transcript:five_prime_UTR. Note that this implementation is subject to change with the introduction of GFF3, which provides a more robust mechanism for grouping features than is possible with the GFF2 specification.

8. The Bio::DB::GFF example script accepts two positional arguments. The first is the method:source of the feature to fetch from the database. The optional second argument acts as a boolean flag to fetch the DNA of the feature in FASTA format. The following command will fetch all exons from *C. elegans*, printing their absolute start and stop positions and their sequence.

```
shell> gff_example.pl coding_exon:Coding_transcript dna
```

9. To utilize the public data-mining server for Bio::DB::GFF scripts, users should set the "dsn" option to " `dbi:mysql:aceserver.cshl.org`," and the username to "`anonymous`." The password field is not required. The example script sets these values automatically.

```
my $db = Bio::DB::GFF->new(-dsn => 'dbi:mysql:aceserver.cshl.org',
-user => 'anonymous');
```

Appendix: Select URLs

The primary WormBase server	http://www.wormbase.org/
Mirror sites:	
Caltech (Pasadena, CA)	http://caltech.wormbase.org/
IMBB (Crete, Greece)	http://imbb.wormbase.org/
Development server	http://dev.wormbase.org/
Data mining server	aceserver.cshl.org
Ports:	Web: http://aceserver.cshl.org/
	Bio::DB::GFF scripts: 3306
	AcePerl scripts: 2005
FTP site	ftp://ftp.wormbase.org/
Acceptable use policy	http://www.wormbase.org/about/ acceptable_use.html
Citing WormBase	http://www.wormbase.org/about/citing.html
Data mining archive	http://www.wormbase.org/data_mining/
Installation guide	http://www.wormbase.org/docs/INSTALL. html
Data submission, questions, comments	help@wormbase.org

Acknowledgments

References

1. Stein, L., Sternberg, P., Durbin, R., Thierry-Mieg, J., and Spieth, J. (2001) WormBase: network access to the genome and biology of *Caenorhabditis elegans*. *Nucleic Acids Res*. **29,** 82–86.

2. Harris, T. W., Lee, R., Schwarz, E., et al. (2003) WormBase: a cross-species database for comparative genomics. *Nucleic Acids Res.* **31,** 133–137.

3. Harris, T. W., Chen, N., Cunningham, F., et al. (2004) WormBase: a multi-species resource for nematode biology and genomics. *Nucleic Acids Res.* **32,** 411–417.

4. Chen, N., Harris, T. W., Antoshechkin, I., et al. (2005) WormBase: a comprehensive data resource for *Caenorhabditis* biology and genomics. *Nucleic Acids Res.* **33,** 383–389.

5. Durbin, R. and Thierry-Mieg, J. (1991) A *C. elegans* database. Documentation and code available from www.acedb.org. Accessed: April 7, 2006.

6. The *C. elegans* Genome Sequence Consortium. (1998) Genome sequence of the nematode *C. elegans*: a platform for investigating biology. *Science* **282,** 2012–2018.

7. Stein, L. D., Bao, Z., Blasiar, D., et al. (2003) The genome sequence of *Caenorhabditis briggsae*: a platform for comparative genomics. *PloS Bio.* **1,** E45.

8. Fraser, A. G., Kamath, R. S., Zipperlen, P., Martinez-Campos, M., Sohrmann, M., and Ahringer, J. (2000) Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. *Nature* **408,** 325–330.

9. Gonczy, P., Echeverri, C., Oegema, K., et al. (2000) Functional genomic analysis of cell division in *C. elegans* using RNAi of genes on chromosome III. *Nature* **408,** 331–336.

10. Kamath, R. S., Fraser, A. G., Dong, Y., et al. (2003) Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* **421,** 231–237.

11. Sonnichsen, B., Koski, L. B., Walsh, A., et al. (2005) Full-genome RNAi profiling of early embryogenesis in *Caenorhabditis elegans*. *Nature* **434,** 462–469.

12. Reboul, J., Vaglio, P., Rual, J. F., et al. (2003) *C. elegans* ORFeome version 1.1: experimental verification of the genome annotation and resource for proteome-scale protein expression. *Nat. Genet.* **34,** 35–41.

13. Lamesch, P., Milstein, S., Hao, T., et al. (2004) *C. elegans* ORFeome version 3.1: increasing the coverage of ORFeome resources with improved gene predictions. *Genome Res.* **14,** 2064–2069.

14. Sulston, J. E. and Horvitz, H. R. (1977) Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. *Dev. Biol.* **56,** 110–156.

15. Sulston, J. E., Schierenberg, E., White, J. G., and Thomson, J. N. (1983) The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **100,** 64–119.

16. White, J. G., Southgate, E., Thomson, J. N., and Brenner, S. (1986). The structure of the nervous system of *Caenorhabditis elegans*. *Philos. Trans. R. Soc. Lond. (B. Biol. Sci.)*. **314,** 1–340.

17. Additional information on BioMart is available at http://www.ebi.ac.uk/biomart/. Accessed: April 7, 2006.

18. Stein L. D. and Thierry-Mieg, J. (1998) Scriptable access to the *Caenorhabditis elegans* genome sequence and other ACEDB databases. *Genome Res.* **8,** 1308–1315.

19. Stajich, J. E., Block, D., Boulez, K., et al. (2002) The BioPerl toolkit: Perl modules for the life sciences. *Genome Res.* **12,** 1611–1618.

20. Stein, L. D., Mungall, C. Shu, S., et al. (2002) The generic genome browser: a building block for a model organism system database. *Genome Res.* **12,** 1599–1610.
21. Dowell, R. D., Jokerst, R. M., Day, A., Eddy, S. R., and Stein L. (2001) The distributed annotation system. *BMC Bioinformatics* **2,** 7 Epub, Oct 10.
22. Kent, W. J., and Zahler, A. M. (2000) Conservation, regulation, synteny, and introns in a large-scale *C. briggsae–C. elegans* genomic alignment. *Genome Res.* **8,** 1115–1125.
23. Schwarz, R.L. and Christiansen, T. (2001). *Learning Perl, 2nd Ed.*, O'Reilly, Sebastapol, CA.

4

C. elegans Deletion Mutant Screening

Robert J. Barstead and Donald G. Moerman

Summary

The methods used by the *Caenorhabditis elegans* Gene Knockout Consortium are conceptually simple. One does a chemical mutagenesis of wild-type *C. elegans*, and then screens the progeny of the mutagenized animals, in small mixed groups, using polymerase chain reaction (PCR) to identify populations with animals where a portion of DNA bounded by the PCR primers has been deleted. Animals from such populations are then selected and grown clonally to recover a pure genetic strain. We categorize the steps needed to do this as follows: (1) mutagenesis and DNA template preparation, (2) PCR detection of deletions, (3) sibling selection, and (4) deletion stabilization. These are discussed in detail in this chapter.

Key Words: *Caenorhabditis elegans*; DNA template preparation; mutagenesis; polymerase chain reaction; mutation discovery; deletion allele; reverse genetics; gene knockout.

1. Introduction

RNAi is now used routinely to discover the loss of function phenotypes for *Caenorhabditis elegans* genes. Whether or not RNAi is used successfully, however, the next step is to recover a genetic mutant, sometimes to confirm the RNAi result, and always to set up the next phase of genetic experimentation. With *C. elegans*, several methods may be used to go from gene to mutant *(1–8)*. Surprisingly, methods to do this via homologous recombination were only recently developed *for C. elegans* and consequently, at present, one finds few citations in the literature that report the use of this method *(2,7)*. A robust but less direct method to go from gene to mutant, used by the *C. elegans* Gene Knockout Consortium and the Japanese National Bioresource Project uses polymerase chain reaction (PCR) in screens to discover animals with deletions of specific target DNA *(6,8)*. Although this method is best suited for large genome scale resource projects, it has also been very effective when used *ad*

hoc by individual investigators. It is most appropriately used when one has multiple targets, any of which would provide an entrée to a pathway.

2. Materials

1. Rich nematode growth medium (RNGM): For 1 L of RNGM combine 3 g NaCl, 7.5 g bacto peptone, and 15 g agarose. Autoclave and cool to 55°C. Add 1 mL cholesterol (5 mg/mL in ethanol; **Caution:** flammable), 1 mL 1 M CaCl$_2$, 1 mL 1 M MgSO$_4$, and 25 mL 1 M KH$_2$PO$_4$, pH 6.0. The peptone is 3X standard NGM. We use agarose to inhibit the burrowing of worms. Not all brands of agarose work for this purpose. We recommend Electrophoresis Grade Agarose from Fisher Biotech (cat. no. BP160-500).
2. 2X YT bacterial growth medium: For 1 L: combine 900 mL of distilled H$_2$O, 16 g bacto tryptone, 10 g bacto yeast extract, and 5 g NaCl. Adjust pH to 7.0 with 5 N NaOH. Add distilled H$_2$O to bring final volume to 1 L. Sterilize by autoclaving.
3. M9 buffer: 22 mM KH$_2$PO$_4$, 22 mM Na$_2$HPO$_4$, 85 mM NaCl, and 1 mM MgSO$_4$.

3. Methods

3.1. Culturing Worms for Mutagenesis

Because chemicals do not cause deletions directly, but rather cause lesions in the DNA that disrupt the DNA repair or replication machinery thereby leading to a deletion, the most appropriate stage for exposure to the mutagen is during the period where the target of the mutagenesis, the DNA in the germ line, undergoes replication. In *C. elegans,* this is during the third and fourth larval stages (L3/L4) when the germ line undergoes mitotic expansion prior to meiosis.

1. To isolate large numbers of L3/L4 staged animals, grow worms on 5–10 15-cm RNGM plates to generate a large population of gravid adults.
2. Wash the gravid adults off of the plates and treat with basic hypochlorite to harvest the eggs.
3. Collect worms by centrifugation in M9 buffer.
4. Add 10 vol of basic hypochlorite (0.25 M KOH, 1.0–1.5% hypochlorite, freshly mixed), and incubate at room temperature for about 4 min.
5. Collect eggs (and some residue of carcass) by centrifugation (400g, 5 min, 4°C).
6. Wash eggs five times with 10 vol M9 and collect by centrifugation.
7. Distribute eggs across 10–20 15-cm plates made with RNGM and seeded with *Escherichia coli* HB101.
8. After 36 h of growth at 20°C the resulting worms are collected and mutagenized.

3.2. Mutagenesis

We treat the staged larvae with the chemical mutagen trimethylpsoralen (TMP; Sigma Chemical Company). Recommendations in the literature for TMP concentration for the mutagenesis of *C. elegans* vary from a high of 30 µg/mL to a low of 0.5 µg/mL *(6,8–10)*. Early on, our dose–response studies determined that a dose of 2 µg/mL would give a reasonable chance of generat-

ing a mutant and yet yield a strain that was still relatively healthy as measured by brood size (Edgley and Moerman, unpublished). As we gained more experience, however, we discovered that the proper dose for TMP varies from batch to batch and even between bottles in a single batch. At present, therefore, we titrate the TMP by doing several mutageneses with TMP concentrations that range from 2 to 16 µg/mL.

1. Incubate worm suspensions in M9 buffer in a horizontal, capped 50-mL centrifuge tube at room temperature in the dark for 15 min with the appropriate concentrations of TMP.
2. TMP must be crosslinked to the DNA to act as a mutagen. Crosslinking is done with UV light generated by an EFOS Novacure, a high-quality filtered UV light source (*see* **Note 1**). After soaking in TMP, transfer the worms in the dark to a sterile glass 10 cm Petri plate and irradiate the suspension with 360 nm UV light for 60 s 340 mW/cm with gentle shaking (*see* **Note 2** for an alternative to UV/ TMP mutagenesis).
3. Following the UV/TMP mutagenesis, the animals are cultured for an additional 36 h, after which they become adults with eggs.
4. Harvest these eggs, which contain the F1 progeny of the mutagenized animals, with a second treatment with alkaline hypochlorite.
5. After the eggs hatch, culture the resulting L1 larvae on standard NGM plates.
6. When they reach the L4/young adult stage, they are used to construct a screening library. Choose a population of worms that exhibits some lethality but still shows a healthy brood size.

3.3. Library Culture

1. L4/young adult worms are cultured in standard bacteriological media 2X YT containing 2% (w/v) *E. coli* (grown separately) and 5 µg/mL cholesterol.
2. Deposit worms into 96-well microplate cultures using a TiterTek Multidrop, an eight-channel liquid dispensing system that pumps the animals from a common reservoir and dispenses them into a 96-well microtiter plate. On average, we dispense 25 diploid animals, representing 50 mutagenized genomes, into each microtiter well. Using this distribution system, we see about a 40% well-to-well variation in the numbers of worms. The final culture volume is 45 µL/well (*see* **Note 3** regarding problems with the library culturing).
3. Typically, we establish 4608 populations, or the number required to fill forty-eight 96-well microtiter plates. We allow these initial pools to grow for 6 d, after which most of the animals in each population represent the F2 descendants from the initial F1 starting population. During the culture period, the animals are held at 20°C in a humidified chamber (*see* **Note 4**).

3.4. DNA Template Preparation

1. Harvest 33% of each population and prepare DNA for PCR. The 66% of the worms that remain after the harvest are reserved to provide live animals from which to recover the desired mutation.

2. Transfer harvested worms to a fresh microtiter well and add an equal volume of a solution containing worm digestion buffer: 50 m*M* Tris-HCl, pH 8.4, 1 m*M* EDTA, 0.5% Tween-20, 0.5% NP-40, 200 µg/mL proteinase K.

3. Close the filled microtiter plates using a foil heat seal and then incubate at 55°C for 3 h with constant shaking in a dedicated microplate shaker (ATR Systems).

4. Inactivate proteinase K by heating to 85°C for 30 min.

3.5. PCR Detection of Deletions

The mutant libraries contain 4608 sample populations. Rather than testing all of these by PCR individually, we strategically pool the samples in a way that balances the requirements for the sensitivity of the assay with our desire to identify the particular source of the positive sample with a minimal number of PCRs. In one pooling strategy, we collect and pool the samples from one microtiter plate into a single sample. The initial screening PCRs, done in duplicate on the pooled samples, lead to a plate address. All 96 samples from the positive plate are then tested individually to arrive at a sample address.

To detect deletions at a target locus, we either do standard PCR with nested primers (*6*) or we do what is called the "poison primer" method (*5*). With the standard method, we do PCR in two rounds with a nested set of primers that produce a wild-type PCR fragment of about 2–3 kb. For poison primer, we add a third primer to the first round of amplification. The third primer is chosen to fall within the target region. With a third primer in the reaction, amplification from the wild-type template leads to the production of two fragments, one full-length and one relatively short. In practice, the shorter fragment is produced much more efficiently than the longer. Amplification from a mutant template, in which the site for the third internal primer is deleted, leads to the production of a single mutant fragment from the normal external primers. In the second round of PCR, we use two primers placed just inside the external first round primers. The shorter wild-type band from the first round cannot serve as a template for the second-round PCR because it does not include one of the second round primer sites. The longer wild-type fragment can serve as a template, but because its production was limited by competition in the first round, its production in the second round is limited correspondingly. The lower level of effective wild-type gives the deletion fragment an advantage. With either of these methods we use a web-enabled software package called Aceprimer to identify appropriate primers to detect deletions in any given gene (http://elegans.bcgsc.bc.ca/gko/aceprimer.shtml).

There are two advantages to the poison primer method. Compared with the standard method poison primer is more sensitive for small deletions. Second, poison primer gives increased precision over the position of the deletion within

the target. This is especially useful for small genes like those that encode micro RNAs. The disadvantage of poison primer, relative to our standard conditions, is that the target is restricted to the position of the poison primer, and so the overall yield of mutations is somewhat lower.

1. PCR reactions are performed in 96-well PCR trays sealed with dimpled rubber mats (Perkin Elmer 96-well plate cover, cat. no. N801-0550). The first round reaction conditions are as follows: for a 25-μL reaction: 5 μL template DNA, 10 pM of each outside primer, 2.5 μL of a 10X dNTP stock (10X = 2 mM), 2.5 μL of a 10X assay buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 25 mM MgCl$_2$), 0.5 U of Taq polymerase (Roche Diagnostics/Boehringer Mannheim), 94°C, 30 s, 1 cycle, 92°C, 30 s; 55°C, 20 s; 72°C, 2 min; 35 cycles.

2. For the second round of the nested PCR reactions we duplicate the first round conditions substituting the appropriate nested primers. The template DNA for the second-round reaction is transferred from the first round reaction using a 96-pin replicator (Boekel Replicator, Fisher, cat. no. 05-450-9). The replicator transfers an insignificant volume to the reaction and so the reaction mix must be adjusted appropriately. If you insist on using a micropipet to transfer the first round to mix to the second round reaction, you must dilute the first round reaction 5- to 10-fold.

3. Putative deletions are identified by the presence of a PCR fragment that is shorter than the wild-type fragment. Fragment sizes are assessed electrophoretically on 1% agarose gels. The gels are configured to accept samples from a standard 96-well PCR plate and are loaded with a 12-channel micropipet.

3.6. Sibling Selection

Once a well of worms is identified and confirmed, it is picked for sibling (sib) selection. At this stage the library cultures are completely starved. The goal is to recover as many worms as possible from the well, and set them up on new food (constituting first-round sib selection, or sib 1). Worms from a positive well are transferred to 60-mm seeded NGM agar or agarose plates for 1 d to allow recovery from starvation and to separate live worms from corpses (which may contribute a positive PCR signal but not allow isolation of viable mutant animals).

1. To culture the worms for sib 1, grow a flask of HB101 in 2X YT/cholesterol. This medium is used with the pregrown bacteria to recover the worms from the 60-mm plate culture.

2. Using a manual 12-channel micropipet and following a limiting dilution, dispense the worms at approximately eight per well to 96-wells of a new microtiter plate.

3. Hold these cultures at 20°C in a humidified box for 5 d.

4. As with the library screens, harvest a portion of each culture and prepare DNA for PCR.

5. When positive wells are identified at sib 1, up to eight wells are recovered to be dispensed as single-worm second-round sibs (sib 2). Once again, positive sib 1 cultures are transferred to 60-mm seeded NGM agar or agarose plates.

6. After 2 d, using sterile toothpicks, pick single worms into a medium of 1X YT/ cholesterol that contains precultured *E. coli* HB101. Worms are picked without regard to phenotype or larval stage.

3.7. Deletion Stabilization

We attempt to generate a homozygous strain for each deletion mutant. Testing for homozygosity, however, is complicated by the fact that the PCR phenotype is dominant so that we detect a deletion band in both heterozygous and homozygous animals (*see* **Note 5**). One might expect that heterozygous and homozygous animals could be easily identified because the PCR assay would show both wildtype and mutant fragments. In practice, however, owing to its smaller size the mutant PCR fragment competes with and ultimately obliterates the production of the wild-type fragment. Using our typical PCR assay, therefore, we cannot always discriminate between homozygous and heterozygous animals. We, therefore, use genetic segregation to test for homozygosity. Sib 2 is the first stage at which we can test whether a deletion line is homozygous.

1. To test for homozygosity, establish 24 new populations from each of four different positive sib 2 populations. If at least 19 or 20 wells of a particular sib 3 set are positive, we conclude that the deletion is probably homozygous viable.

2. Confirm this by testing four more sib 3 populations. Again, 24 single animals are subjected to PCR from each of the four positive sib 3 cultures. We conclude that the strain is homozygous if all 24 picks are positive.
   Some deletion alleles fall within genes that are essential for viability. In such cases, the allele cannot be maintained in a homozygous strain, but rather must be maintained *in trans* to a genetic balancer chromosome. Such balancer chromosomes must be introduced through individual genetic crosses. A list of the balancers we use (including versions marked with pharyngeal GFP insertions) is available at http://ko.cigenomics.bc.ca/balancers.html. For balancers that are not GFP-marked, we have constructed male balancer strains carrying an unlinked chromosomal GFP insertion homozygous in the background, which allows unequivocal identification of cross-progeny. Once the balanced line is constructed and identified, nonGFP balanced heterozygotes are selected from it to establish the final strain population. A general strategy for balancing is as follows:

3. If a deletion is suspected to be lethal as a homozygote (i.e., it has not been possible to isolate homozygotes in four rounds of sib-selection), the line is picked for a balancing cross. Four positive populations are transferred to new seeded 60-mm NGM plates and allowed to recover for 2 d.

4. Select five or six of the oldest but still fertile hermaphrodites from each plate for crosses with balancer males. If cross-progeny can be identified by virtue of GFP, then 24–48 animals are picked to establish single populations; if cross-progeny

cannot be so identified, then many L4 hermaphrodite progeny are selected to establish new populations once the first L4 males appear to indicate the cross was successful.

5. After 5 d wells are screened to identify those that segregate the balancer (if the balancer was non-GFP), and then all populations are sampled for PCR to identify those positive for the deletion.

6. Transfer two to four wells positive for both the balancer and the deletion to new plates for closer examination and to set up new clonal populations.

7. Examine progeny from these for segregation of the balancer and for any visible phenotypes attributable to deletion homozygotes and test by PCR to confirm presence of the deletion.

4. Notes

1. An alternative UV light source can be constructed by mounting two hand-held UV lamps (model UVL-21 Blak-Ray Lamp, longwave UV-365 nm, Fisher Scientific, cat. no. 11-984-40) using appropriate clamps on a ring stand. We adjust the height of the lamps to generate the appropriate UV dose as measured with an UV dose meter (model UVX Digital Radiometer with UVX-36, Fisher Scientific, cat. no. 97-0015-01).

2. Recently, we have tested the well-characterized mutagen ethylmethanesulfonate (EMS) as an alternative to UV/TMP (*9*). Libraries made using EMS as the mutagen show a slightly lower yield of mutants, but the overall health of the resulting strains is somewhat better compared to mutants made by UV/TMP. We use EMS as described by Anderson (*9*). To an 8-mL suspension of worms in M9 buffer we add 8 mL of M9 containing 100 mM methane sulfonic acid ethyl ester (Sigma, cat. no. M-0880) to yield a final EMS concentration of 50 mM. We incubate the worm suspension in a horizontal, capped 50-mL centrifuge tube at room temperature for 4 h with moderate shaking. We then wash the worms five times with 10 vol of M9 buffer. After each wash the worms are collected by centrifugation at 1000g at 4°C for 5 min.

3. Microbial contamination is a serious problem with mutant library production. Contamination can enter the system either through manipulations of the worms or (because the worms are fed *E. coli*) through a contaminant introduced as the *E. coli* is cultured. One can reduce the chance for contamination through proper staff training, and through the liberal use of hand wash stations and spray disinfectants in all the work areas.

4. Because the worm cultures are fed live, respiring *E. coli*, the cultures sometimes become anoxic. In such cases, the worms suffer more than the *E. coli*. This has a negative impact on both the initial identification of deletion mutants, and the ability to recover worms from the targeted culture in the downstream steps. To some extent, underfeeding the cultures avoids this problem. A better solution, if available, is to culture the worms in a humidified, microplate shaker–incubator.

5. In general, the procedures previously outlined, in which we follow the deletion band until we obtain a homozygous animal, are sound. However, occasionally

(<5% according to current data), mutagenesis for library construction results in an apparent deletion/duplication event, such that a deleted copy of the gene is present in the genome in addition to a functional wild-type copy. The presence of a wild-type copy of the gene is not detectable by our standard PCR procedures because in the vast majority of cases the reaction favors the deleted product to the extent that the wild-type product is not present. Such events may not result in a mutant phenotype.

References

1. Rushforth, A. M., Saari, B., and Anderson, P. (1993) Site-selected insertion of the transposon Tc1 into a *Caenorhabditis elegans* myosin light chain gene. *Mol. Cell. Biol.* **13,** 902–910.
2. Berezikov, E., Bargmann, C. I., and Plasterk, R. H. (2004) Homologous gene targeting in *Caenorhabditis elegans* by biolistic transformation. *Nucleic Acids Res.* **32,** e40.
3. Jansen, G., Hazendonk, E., Thijssen, K. L., and Plasterk, R. H. (1997) Reverse genetics by chemical mutagenesis in *Caenorhabditis elegans*. *Nat. Genet.* **17,** 119–121.
4. Liu, L. X., Spoerke, J. M., Mulligan, E. L., et al. (1999) High-throughput isolation of *Caenorhabditis elegans* deletion mutants. *Genome Res.* **9,** 859–867.
5. Edgley, M., D'Souza, A., Moulder, G., et al. (2002) Improved detection of small deletions in complex pools of DNA. *Nucleic Acids Res.* **30,** e52.
6. Barstead, R. J. (1999) Reverse genetics. In: C. elegans*: A Practical Approach,* (Hope, I., ed), Oxford University Press, New York, NY, pp. 97–141.
7. Barrett, P. L., Fleming, J. T., and Gobel, V. (2004) Targeted gene alteration in *Caenorhabditis elegans* by gene conversion. *Nat. Genet.* **36,** 1231–1237.
8. Gengyo-Ando, K. and Mitani, S. (2000) Characterization of mutations induced by ethyl methanesulfonate, UV, and trimethylpsoralen in the nematode *Caenorhabditis elegans*. *Biochem. Biophys. Res. Commun.* **269,** 64–69.
9. Anderson, P. (1995) Mutagenesis. *Methods Cell. Biol.* **48,** 31–58.
10. Yandell, M. D., Edgar, L. G., and Wood, W. B. (1994) Trimethylpsoralen induces small deletion mutations in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* **91,** 1381–1385.

5

Insertional Mutagenesis in *C. elegans* Using the *Drosophila* Transposon *Mos1*

A Method for the Rapid Identification of Mutated Genes

Jean-Louis Bessereau

Summary

One benefit of the nematode *Caenorhabditis elegans* as a model system is the ease to conduct forward genetic screens and to isolate mutants with phenotypes of interest. However, identifying the mutated genes requires positional cloning, which can be laborious and time consuming. Insertional mutagenesis with a heterologous transposon bypasses the mapping steps and expedites the process of identifying the mutated genes. The *Drosophila* transposon *Mos1* can be mobilized in the *C. elegans* germline to cause mutations. Mutagenic insertions are subsequently localized within the genome using inverse polymerase chain reaction. The mutagenicity of this technique is roughly one order of magnitude lower than chemical mutagens. However, the molecular identification of the mutated genes is extremely rapid. Therefore, before using *Mos1*-mediated mutagenesis, one must evaluate the trade-off between time spent screening for mutants vs time spent mapping and rescuing a mutation.

Key Words: *Caenorhabditis elegans*; insertional mutagenesis; transposon; *Mos1*; inverse PCR.

1. Introduction

Genetic features of the nematode *Caenorhabditis elegans* make this organism particularly advantageous to conduct forward genetic screens (*1,2*). Under laboratory conditions, most animals are diploid hermaphrodites which reproduce by self-fertilization with a generation time of approx 3.5 d at 20°C. Self-fertilization has several advantages. First, mutagenizing a limited number of founder animals will generate at the subsequent generation a large population of individuals carrying mutations in one gene copy at random loci. In most cases, a heterozygous mutant will in turn generate homozygous mutants by

self-fertilization, as one-quarter of its progeny according to Mendelian laws. Hence, phenotypes owing to recessive mutations can be looked for directly in the second generation of mutagenized animals without requiring any specific cross. Second, recessive mutations causing lethal or sterile phenotypes can be maintained in heterozygous animals. Third, mutants exhibiting severe anatomical or behavioral phenotypes can be maintained as homozygous stocks because they do not need to mate for reproduction. Fourth, the use of sensitized genetic backgrounds is especially powerful in *C. elegans* because it is possible to mutagenize mutant founders and screen directly in the progeny for suppressors and enhancers of the initial mutation present in the background.

Chemical mutagens, such as ethylmethanesulfonate (EMS), have been extensively used because they are easy to utilize, extremely efficient, and generate a wide range of mutations. However, identification of the mutated genes requires positional cloning. This step can be extremely laborious and time consuming, especially when mapping synthetic phenotypes or when the mutant phenotype is very subtle. Single-nucleotide polymorphism mapping techniques provide a significant improvement in the speed of positional cloning *(3)*, but it is still difficult to perform on certain phenotypes because polymorphic strains have exhibited phenotypic differences relative to the reference Bristol laboratory strain *(4)*.

Insertional mutagenesis with a transposon circumvents the need for genetic mapping: the transposon can be used as a sequence tag to rapidly identify the mutated gene. Endogenous transposable elements of the *Tc1/mariner* superfamily, especially *Tc1* and *Tc3*, have been widely used for insertional mutagenesis in *C. elegans (5–8)*. However, using *Tc* elements as mutagens has some major drawbacks. First, the mobilization of *Tc* transposons is achieved in mutator strains, which activate multiple classes of transposable elements *(9–11)*. Second, all known isolates of *C. elegans* contain multiple copies of *Tc* elements, which complicate the identification of the mutagenic insertion. Third, in the mutator strains that are used, transposition is not controlled. Some *Tc* insertions are poorly mutagenic either because they are in introns, or because they are removed from the mature mRNA by aberrant splicing *(12,13)*. Spontaneous re-excision can generate mutagenic footprints that generate a stronger phenotype but can no longer be detected in a transposon tagging strategy. These limitations have been circumvented by mobilizing the *Mos1* transposon in the germ line of *C. elegans (14,15)*.

Mos1 is a member of the *Tc1/mariner* family and was isolated from *Drosophila mauritiana (16)*. Like all *Tc1/mariner* elements, *Mos1* transposes by a conserved cut and paste mechanism *(17,18)*. It contains a single gene encoding a transposase that is flanked by short terminal inverted repeats. The transposase enzyme binds to the inverted repeats and catalyzes the excision of an element from the genome and subsequent insertion at a new genomic loca-

tion. In *C. elegans*, a two-component system was developed to mobilize *Mos1* in the germline (*14*). The first component is an extrachromosomal array, which expresses the transposase enzyme under the control of a heat-shock promoter, and the second component is another transgene, which carries the substrate transposon. Double transgenic hermaphrodites are subjected to heat shock to activate the heat-shock promoter and thereby express the transposase enzyme. In turn, the transposase enzyme catalyzes the transposition of a *Mos1* element from the substrate array into a chromosomal location. Progeny of heat-shocked double transgenic parents are subsequently screened for a phenotype of interest at the F1 or F2 generation. After mutant lines have been established, *Mos1* insertions are localized within the genome by polymerase chain reaction (PCR). The average number of insertions is 2.5 but ranges from 1 to 10 (**ref. *15*** and unpublished results). In cases of multiple insertions, it is possible to identify which insertion is causing the mutant phenotype either by serial outcrossing of the mutant strain to lose nonmutagenic insertions or by looking for linkage between specific insertions and the mutant phenotype.

In the most favorable situations, it is possible to go from a mutant phenotype to the identity of the mutated gene within 2 wk. However, *Mos1*-mediated mutagenesis is not as robust as EMS mutagenesis and one must consider several points before deciding to use *Mos1*. First, mobilizing *Mos1* is not as robust as exposing worms to EMS and requires one to pay attention to details all along the course of the procedure. Second, *Mos1*-mediated mutagenesis is about 10 times less efficient than chemical mutagenesis. This is not an issue for easy screens such as selection screens in which the number of screenable animals is almost unlimited. By contrast, *Mos1*-mediated mutagenesis is not recommended for painful screens such as clonal screens looking for the mislocalization of a green fluorescent protein (GFP) under a compound microscope. Third, most mutations caused by transposon insertions are loss-of-function mutations. Therefore, before starting a *Mos1*-mediated mutagenesis, it is highly recommended to perform a small-scale pilot screen using chemical mutagens to verify that mutants of interest are generated at a frequency compatible with standard loss-of-function mutations.

In conclusion, the choice of *Mos1*-mediated mutagenesis will depend on the trade-off between time spent screening for mutants vs time spent mapping and rescuing a mutation caused by a chemical mutagen.

2. Materials

2.1. Worm Strains and Handling

1. Strain EG2762 *oxEx166[hsp::MosTransposase; unc-122::gfp; lin-15(+)].* This strain contains an extrachromosal transgene driving the expression of the Mos transposase under the control of a heat-shock promoter (**Note 1**). The EG2762

strains segregate a mixture of nontransgenic and transgenic animals. Transgenics are identified based on expression of the GFP in coelomocytes.

2. Strain EG1470 *oxEx229[Mos1; myo-2::GFP]* contains an extrachromosal transgene with multiple wild-type copies of *Mos1*. Transgenic animals are recognized based on GFP expression in the pharynx.

3. These strains can be requested, at no expense, at the *Caenorhabditis* Genetics Center (http://biosci.umn.edu/CGC/CGChomepage.htm). Propagate the strains at 25°C (**Note 2**) and freeze multiple vials of the strains on receipt.

4. Dissecting microscope equipped with epifluorescence to visualize GFP in the worms.

5. Standard equipment for *C. elegans* culture.

2.2. Molecular Biology

1. PCR thermocycler.
2. Worm lysis buffer: 50 m*M* KCl, 10 m*M* Tris-HCl, pH 8.3, 2.5 m*M* MgCl$_2$, 0.45% Tween-20, 0.45% NP-40, 0.01% gelatin, and 1 mg/mL proteinase K (added fresh).
3. Oligonucleotides:
   a. oJL102: CAACCTTGACTGTCGAACCACCATAG
   b. oJL103: TCTGCGAGTTGTTTTTGCGTTTGAG
   c. oJL114: AAAGATTCAGAAGGTCGGTAGATGGG
   d. oJL115: GCTCAATTCGCGCCAAACTATG
   e. oJL116: GAACGAGAGGCAGATGGAGAGG
   f. IPCR1a: GACCTTGTGAAGTGTCAACCTTGACTG
   g. IPCR1b: GACAATCGATAAATATTTACGTTTGCGAGAC
   h. IPCR2b: CATCTATATGTTCGAACCGACATTCCC
4. Device for DNA electrophoresis in agarose gels.
5. Gel purification kits, restriction enzymes, modification enzymes, and Taq polymerase are from standard suppliers.

3. Methods
3.1. Mutagenesis by Mobilization of Mos1 in the Germline

The mobilization of *Mos1* relies on two extrachromosomal arrays, one expresses the transposase enzyme (the "enzyme array"), and the other carries the substrate transposon (the "substrate array") (*14,15*). The "enzyme array" *oxEx166* is extrachromosomal. It contains the coding region of the *Mos1* transposase under the control of a heat-shock promoter and is marked by *Punc-122::GFP*, which drives expression of GFP in coelomocytes. The "substrate" array *oxEx229* is also extrachromosomal. It contains multiple copies of the *Mos1* transposon and is marked by *Pmyo-2::GFP,* which drives GFP expression in the pharynx. These two arrays were generated independently and are maintained in separate strains. To mobilize *Mos1*, males carrying the enzyme array are crossed to hermaphrodites with the substrate array to generate double transgenic hermaphrodites that contain both arrays. Double transgenic hermaphrodites are subjected to heat

shock to activate the heat-shock promoter and thereby express the transposase enzyme. In turn, the transposase enzyme catalyzes the transposition of a *Mos1* element from the substrate array into a chromosomal location. Progeny of heat-shocked double transgenic founders are subsequently screened for a phenotype of interest at the F1 or F2 generation (**Fig. 1**).

1. Set up two mating plates with 10 N2 males and three *oxEx166* hermaphrodites per plate. Put the plate at 25°C (**Note 2**).
2. From this cross, pick males with fluorescent coelomocytes under fluorescence microscope and use these males to set up three to five mating plates with *oxEx229* transgenic hermaphrodites. Keep the plates at 25°C (**Note 2**).
3. From these crosses, pick double transgenic larval hermaphrodites containing GFP in pharynx and coelomocytes (**Note 3**). Put three double transgenic per plate at 25°C and let them self-fertilize.
4. In the progeny, pick the double transgenics (**Note 3**). They represent the TT.1 generation (transposase and transposon kept together for one generation). Dispense three TT.1 animals per plate and keep the plates at 25°C.
5. From the self-progeny of TT.1 animals, pick TT.2 double transgenics. If this is the first time that you are using the *oxEx166* and *oxEx229* strains, set apart five TT.2 animals to test transposition rate (*see* **Subheading 3.2.**). The other TT.2 animals are used to amplify the population of double transgenic animals. Dispense three TT.2 animals per plate and keep the plates at 25°C.
6. Keep repeating **step 4** to amplify the double transgenic population. Depending on the number of haploid genomes that you want to mutagenize, you might go to the TT.4 to TT.6 generation (**Note 4**).
7. When you have enough double transgenic animals, transfer the *young adults* to a fresh plate (**Note 5**). These animals represent the P0 population that will be heat-shocked. Seal the plate containing the P0s with two layers of Parafilm. Dip the plate in a water bath at 33°C for 1 h. Take the plate out of the water bath and leave it for 1 h at 20°C. Put the plate again at 33°C for 1 h. Take the plate out of water, remove the Parafilm and put the plate at 15°C for 14–18 h.
8. Transfer P0s at 20°C and let them lay eggs. The highest transposition frequency is observed in F1s laid within 24 h after transfer of the heat-shocked plate at 20°C. Set up your screen as you would do with animals mutagenized with EMS.
9. When the population of F1 animals is grown, set apart 24 individual F1s on single plates for subsequent measurement of the transposition rate that was obtained in the heat-shocked P0s (*see* **Subheading 3.2.**).
10. Screen the F1 or F2 population for the phenotype you are interested in.
11. Clone individual mutants to establish mutant lines.

3.2. Measuring Transposition Rate

The mutagenicity of this technique relies on the efficiency of *Mos1* transposition in the germline. This process depends on transgenic arrays that, we know, can be silenced under conditions that have not been clearly identified *(15)*. There-

N2 ♂♂   ✕   oxEx166 ♀♀
                    Green coelomocytes

@ 25°C

oxEx166 ♂♂   ✕   oxEx229 ♀♀
Green coelomocytes   Green pharynx

@ 25°C

oxEx166; oxEx229 ♀♀
Green coelomocytes + pharynx

Self @ 25°C

TT.1 generation
(**Transposase** and **Transposon** kept together for **1** generation)
Green coelomocytes + pharynx

Self @ 25°C

TT.2
Green coelomocytes + pharynx

HEAT SHOCK 10 ♀♀

TEST TRANSPOSITION RATE
(if strains have not been tested in the lab
or if they have been propagated for more
than two months)

AMPLIFY THE POPULATION OF
DOUBLE TRANSGENICS

Self @ 25°C

TT.4 to .6
=
P0 population

HEAT SHOCK YOUNG ADULTS
Self @ 20°C

Clone 24 individuals   ◄——— **F1 population**
to measure transposition frequency.

F2 population

Fig. 1. Overall strategy of *Mos1*-mediated mutagenesis (*see* **Subheading 3.1.**). The F1 or F2 populations will be screened for mutant phenotypes depending of the specific design of the screen.

fore, it is essential to make sure that the strains that you propagate in your laboratory promote efficient transposition. In addition, a control of transposition rate must be performed during the screen to control the efficacy of the mutagenesis step. After heat-shocking double transgenic hermaphrodites, the transposition

frequency is defined as the fraction of the progeny that contains at least one genomic *Mos1* insertion, i.e., animals that lost the substrate array *oxEx229[Mos1; myo-2::GFP]* and are positive for *Mos1* by PCR. The routine rate is 50 ± 15%. If this rate is less than 30%, something is wrong. To measure the transposition rate, proceed as follows:

1. Heat-shock double transgenic young adults and let them lay F1s as in **Subheading 3.1.**, **steps 6** and **7**.
2. Clone 24 individual F1s under visible light. Do not select for animals that already lost the *oxEx229* array at this step because the transposition frequency is lower in this population. You want to sample the F1 generation "blind."
3. From each plate, pick 5-10 F2s that lost *oxEx229*, i.e., have no GFP in the pharynx (the presence of *oxEx166*, i.e., fluorescent coelomocytes does not matter) and put these F2s together on a subsequent plate (**Note 6**).
4. The day after, verify that the F3 larvae do not express GFP in the pharynx. Pick 10–20 larvae into 10 µL of worm lysis buffer. Use *oxEx229* larvae as a positive control and N2 larvae as negative control.
5. Freeze the worms at least 30 min at –80°C. Then incubate for 1 h at 65°C followed by 15 min at 95°C. Put the lysate on ice.
6. Use 1 µL of the total worm lysate for the PCR reaction using the primers oJL102–oJL103. Use a 1.5 m*M* MgCl$_2$ concentration and perform 30 cycles: 45" at 94°C/1' at 56°C/45" at 72°C.
7. Run the PCR product on a 2% agarose gel. The fragment amplified from *Mos1* is 355 bp long. Calculate the transposition frequency by dividing the number of *Mos1(+)* clones by the total number of animals that were analyzed.

3.3. Identifying the Mutagenic Insertion

Mos1 insertion is a molecular tag that enables the rapid identification of the mutated gene. However, there are two concerns to keep in mind. First, mobilization of transposons can generate mutants that do not contain a copy of a transposon ("hit and run" events). These events seem to be rare with *Mos1*: among the mutants that were identified in different *Mos1* screens conducted in our laboratory, a *Mos1* insertion was present in 14 of 15 mutated genes *(15)*. However, a larger proportion of such mutants was reported in screens conducted by other laboratories (Mango, S., personal communication). Second, a mutant strain usually contains more than one insertion. The average number of insertions is 2.5 but ranges from 1 to 11. In case of multiple insertions, it is necessary to identify which insertion is causing the mutant phenotype either by serial outcrossing of the mutant strain to lose nonmutagenic insertions or by looking for linkage between specific insertions and the mutant phenotype. This step is facilitated by the fact that *Mos1* insertions are scattered within the genome of a single animal. After identifying the putative mutagenic insertion, it remains essential to confirm the identity of the mutated gene by other criteria, i.e., rescue experiments or phenocopy by RNA interference.
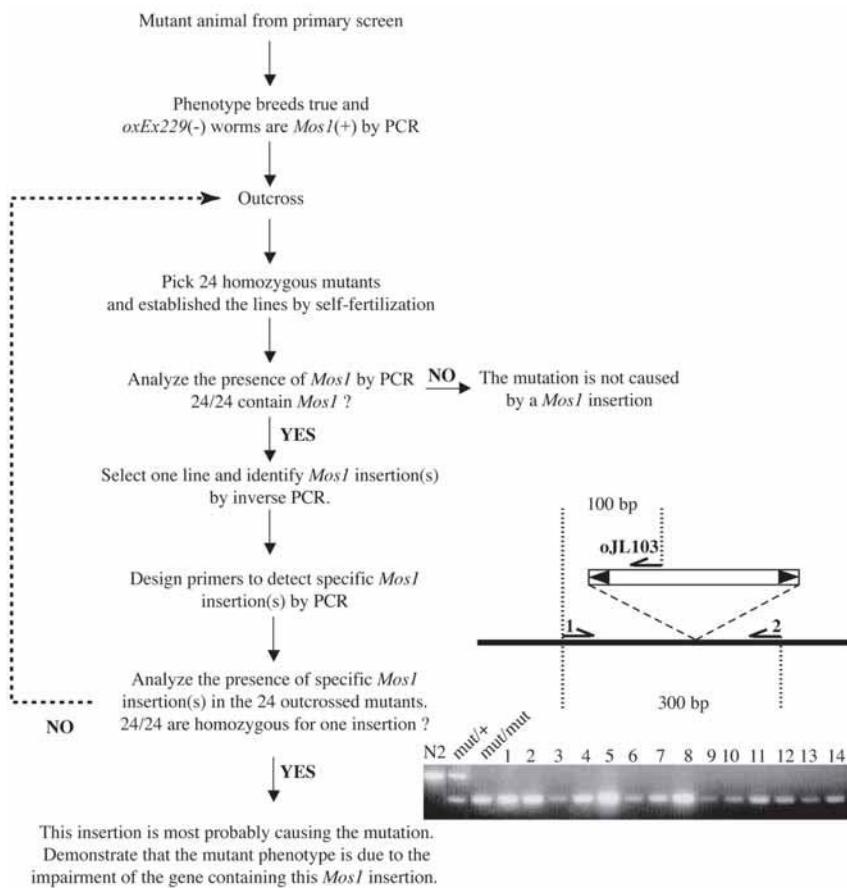
Fig. 2. Overall strategy to identify the *Mos1* insertion causing a phenotype (*see* **Subheading 3.3.**). The right panel shows how primers are designed to detect the presence or not of a *Mos1* insertion at a specific locus. Primers oJL103, 1 and 2 were mixed together in PCR reactions performed on strains of different genotypes. In this example, 14 of 14 outcrossed mutants were homozygous for the *Mos1* insertion, which we subsequently demonstrated to be causing the mutant phenotype.

Here is a possible strategy to identify a mutagenic insertion (summarized in **Fig. 2**):

1. From the mutant cloned in the primary screen, establish a line by self-fertilization and pick animals in the progeny, which lost *oxEx229* (no GFP in the pharynx).
2. Verify that the mutant phenotype breeds true. If not, discard this line.
3. Check the presence of *Mos1* by PCR using oJL102–oJL103 (**Subheading 3.2.**, **steps 4–6**). If the strain is *Mos1*-positive, keep chasing a mutagenic insertion.

4. Cross N2 males with mutant hermaphrodites.

5. Pick four heterozygote L4 hermaphrodites and let them reproduce by self-fertilization.

6. At the subsequent generation, clone 24 mutants and analyze their progeny by PCR for the presence of *Mos1* (**Subheading 3.2.**, **steps 4–6**). All of the 24 outcrossed mutants must contain *Mos1*. If not, the mutation is not caused by *Mos1*. If yes, store the worm lysates at –20°C (or –80°C for longer conservation) and keep the independent outcrossed lines as starved plates.

7. Select one outcrossed line to localize *Mos1* insertion(s) by inverse PCR (*see* **Subheading 3.4.**).

8. After you have localized *Mos1* insertion(s) in the genome, design PCR primers located on each side of the *Mos1* insertion(s). In combination with oJL103, this allows one to differentiate between loci that do or do not contain a *Mos1* insertion (**Fig. 2**; **Note 7**).

9. Analyze the 24 outcrossed mutant lines from **step 6** for the presence of specific *Mos1* insertion(s). If all of the 24 outcrossed homozygous mutants are homozygous for a specific insertion, this means that this insertion is less than two genetic map units away from the actual mutation. In our experience, we never had two independent insertions located in such a small genetic interval. Therefore, one should consider that this specific insertion is causing the mutation. Additional classical strategies (rescue experiments, phenocopy by RNAi, identification of mutations in other alleles, and others) will confirm that the phenotype of interest is caused by the mutation of the gene that contains the *Mos1* insertion.

10. If no insertion is linked to the mutant phenotype, make an additional outcross and redo the procedure starting at **step 6** (**Note 8**). This must be repeated until a *Mos1* insertion linked to the mutant phenotype is identified. Alternatively, identification of an outcrossed mutant that lost all *Mos1* copies based on negative oJL102–oJL103 PCR means that the mutation is not caused by a *Mos1* insertion.

3.4. Localizing Mos1 *Insertions in the* C. elegans *Genome*

Inverse PCR enables the amplification of a short genomic fragment flanking the *Mos1* end (**Fig. 3**). After sequencing the PCR product, the *Mos1* insertion is localized at a single base-pair resolution by comparing the sequence flanking *Mos1* with the *C. elegans* genome. Briefly, genomic DNA from mutants containing *Mos1* insertion(s) is digested with a frequent cutter enzyme. We usually set up two parallel digestions to identify more insertions because some insertions can be missed with a specific enzyme if the closest restriction site in the flanking genomic sequence is too far (no PCR product) or too close (not enough sequence to be informative). After enzyme inactivation, DNA ligase is added to cut genomic DNA. Intramolecular religation is favored. Primers present in the uncut *Mos1* fragment and pointing toward the outer ends of the transposon are used to PCR amplify one *Mos1* end and its flanking genomic region. A second PCR using nested primers is used to generate clean products
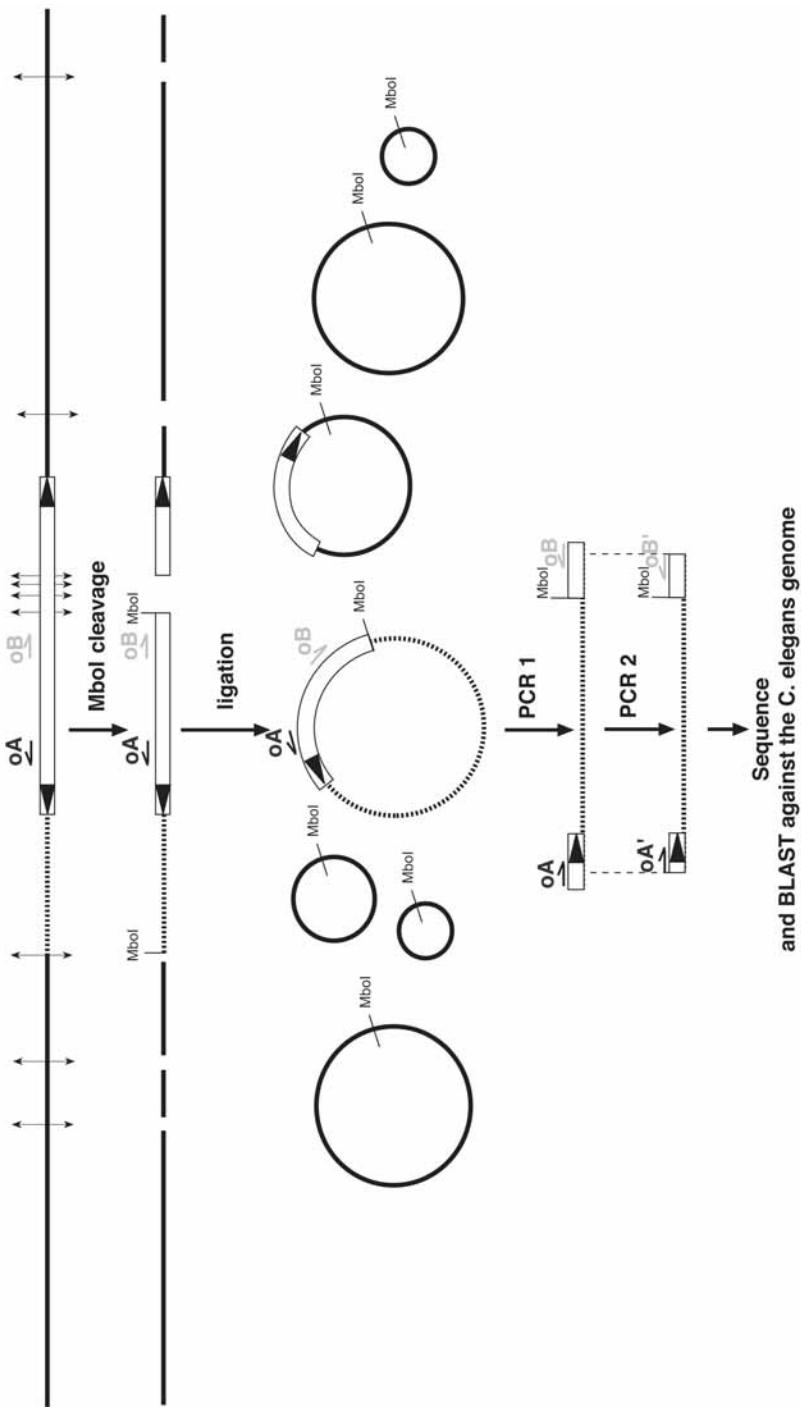
Fig. 3. Localizing *Mos1* insertions in the genome using inverse PCR. In this example, *Mbo*I is used to digest genomic DNA.

to be sequenced (*see* **Fig. 4**). To localize *Mos1* insertion(s) present in a strain, proceed as follows:

1. Put five adult worms into 20 µL of worm lysis buffer.
2. Freeze at least 30 min at –80°C. Then incubate for 1 h at 65°C followed by 15 min at 95°C (**Note 9**).
3. Digest 8 µL of the worm lysate in a final volume of 30 µL using 5 U of one of the following enzymes: *Mbo*I, *Hpa*II, *Hae*III ("Group 1") or *Mse*I, *Alu*I, and *Hha*I ("Group 2"). Subsequent PCR steps will require different sets of primers depending on whether DNA was digested by group 1 or 2 enzymes. As a first choice, we usually choose *Mbo*I and *Mse*I. Incubate 3 h at 37°C (**Note 10**).
4. Inactivate the restriction enzyme by incubation at 75°C for 15 min.
5. Add 10 U of T4 DNA ligase to 10 µL of cut DNA in a final volume of 30 µL. Incubate overnight at 15°C (**Note 11**).
6. Use 3 µL of the ligation for PCR using the primers oJL103–oJL114 if DNA was digested with an enzyme from group 1, IPCR1a–IPCR1b if DNA was digested with an enzyme from group 2. Use a 1.5 m*M* MgCl$_2$ concentration in the PCR reaction and perform 30 amplification cycles: 45" at 94°C, 1' at 60°C, 1' at 72°C.
7. Dilute 1 µL of the PCR reaction in 200 µL water and use 1 µL of the dilution for the subsequent nested PCR. oJL115–oJL116 are used for group 1 enzymes, oJL102–IPCR2b for group 2 enzymes. Use a 1.5-m*M* MgCl$_2$ concentration in the PCR reaction and perform 25 amplification cycles. The annealing temperature is 62°C for oJL115–oJL116 and 59°C for oJL102-IPCR2b.
8. Run the PCR product on a 1.8% agarose gel.
9. Gel-purify candidate bands (**Notes 12** and **13**).
10. Sequence oJL115–oJL116 or oJL102–IPCR2b PCR products using the primers oJL115 or oJL102, respectively (**Note 14**).
11. Identify the location of the *Mos1* insertion using the BLAST tool provided by WormBase (http://www.wormbase.org/db/searches/blat). Paste the sequence in the browser window and run 'BLASTN' against the "*C. elegans* genomic" database. Be aware that some PCR products are fake. To be sure that you are looking at a true insertion, you must read the sequence of the end of the transposon directly followed by the *C. elegans* genomic sequence starting at a TA dinucleotide.
12. Confirm the location of the *Mos1* insertion by designing a primer in the genomic region flanking this insertion and confirm that you can amplify a fragment corresponding to the actual predicted size in combination with oJL103.

4. Notes

1. This strain segregates sick animals (Ste, pVul, Unc,…) and Dpys. These phenotypes never breed true and are most probably caused by leaky expression of the Mos transposase in somatic tissues.
2. We observed that the transposition efficiency can decrease over time. Although not definitely demonstrated, keeping the strains at 25°C might prevent or slow down silencing. Be sure that your incubators are properly regulated because ani-

IPCR2b   IPCR1b

CCAGGTGTACAAGTAGGGAATGTCGGTTCGAACATATAGATGTCTCGCAAACGTAAATA
IPCR1a   oJL102
TTTATCGATTGTCATAAAACTTTGACCTTGTGAAGTGTCAACCTTGACTGTCGAACCAC

CATAGTTTGGCGCGAATTGAGCGTCATAATTGTTTACTCTCAGTGCAGTCAACATGTCG
oJL115
AGTTTCGTGCCGAATAAAGAGCAAACGCGGACAGTATTAATTTTCTGTTTTCATTTGAA

GAAAACAGCTGCGGAATCGCACCGAATGCTTGTTGAAGCCTTTGGCGAACAAGTACCAA

CTGTGAAAACGTGTGAACGGTGGTTTCAACGCTTCAAAAGTGGTGATTTTGACGTCGAC

GACAAAGAGCACGGAAAACCGCCAAAAAGGTACGAAGACGCCGAACTGCAAGCATTATT

GGATGAAGACGATGCTCAAACGCAAAAACAACTCGCAGAGCAGTTGGAAGTAAGTCAAC
oJL103   oJL114
AAGCAGTTTCCAATCGCTTGCGAGAGATGGGAAAGATTCAGAAGGTCGGTAGATGGGTG
oJL116
CCACATGAGTTGAACGAGAGGCAGATGGAGAGGCGCAAAAACACATGCGAAATTTTGCT

TTCACGATACAAAAGGAAGTCGTTTTTGCATCGTATCGTTACTGGAGATGAAAAATGGA

TCTTTTTTGTTAATCCTAAACGTAAAAAGTCATACGTTGATCCTGGACAACCGGCCACA

TCGACTGCTCGACCGAATCGCTTTGGCAAGAAGACGATGCTCTGTGTTTGGTGGGATCA

GAGCGGTGTCATTTACTATGAGCTCTTGAAACCCGGCGAAACGGTGAATACGGCACGCT

ACCAACAACAATTGATCAATTTGAACCGTGCGCTTCAGAGAAAACGACCGGAATATCAA

AAAAGACAACACAGGGTCATTTTTCTCCATGACAACGCTCCATCACATACGGCAAGAGC

GGTTCGCGACACGTTGGAAACACTCAATTGGGAAGTGCTTCCGCATGCGGCTTACTCAC

CAGACCTGGCCCCATCCGATTACCACCTATTCGCTTCGATGGGACACGCACTCGCTGAG

CAGCGCTTCGATTCTTACGAAAGTGTGAAAAAATGGCTCGATGAATGGTTCGCCGCAAA

AGACGATGAGTTCTACTGGCGTGGAATCCACAAATTGCCCGAGAGATGGGAAAAATGTG

TAGCTAGCGACGGCAAATACTTTGAATAAATGATTTTTTCTTTTTCCACAAAATTTAAC

GTGTTTTTTGATTTAAAAAAAACGACATTTCATACTTGTACACCTGA

Fig. 4. *Mos1* sequence and localization of the primers mentioned in this chapter.

mals will become sick and sterile above 25°C. If you encounter sterility or too much sickness during strain propagation, you can decrease the temperature to 23°C or even 20°C.

3. Do not be surprised by the very small number of double transgenic animals arising from such crosses (<10). The fraction of double transgenics will be much greater in the self-progeny of the next generations.

4. The average usable brood size of a heat-shocked double transgenic is comprised of between 10 and 50 F1s. Because transposition most probably occurs only in the female germline of adult animals *(15)*, this corresponds to 10–50 mutagenized haploid genomes.

5. Heat-shock is very toxic for worms that contain both the catalytic and the substrate arrays. The heat-shock promoter is driving high levels of transposase expression in somatic tissues and causes extremely high rates of transposition in somatic cells. We estimate that, on average, 10 copies of *Mos1* insert into each haploid genome in somatic tissues *(14)*. Heat-shock conditions are a compromise between efficiency and toxicity. Heat-shock young adults but not L4 larvae because most L4s will die or be sterile.

6. *Mos1* insertions can be checked by PCR directly on these worms using the primers oJL102–oJL103. However, some of these animals might be mosaic for the *Mos1* array if the parent did contain *oxEx229* and would, therefore, give a false-positive signal.

7. The different PCR conditions usually need to be optimized for the different sets of primers. This can be done using a worm lysate from the outcrossed mutant line used for inverse PCR.

8. Only a fraction of the insertions present in an analyzed strain are detected by inverse PCR. New insertions are usually detected after outcrossing the strain and decreasing the total number of *Mos1* copies in the rare instance of numerous insertions in the same animal.

9. iPCR performed on worm lysates usually works fine. However, we sometimes encountered unexplained failures that could be solved by preparing genomic DNA using the DNAeasy kit™ (Qiagen) according to the manufacturer's recommendations. We used about 150 ng of purified genomic DNA for subsequent steps.

10. Digestion can be controlled by loading 10 μL of the mix on a 1% agarose gel. You expect to see the disappearance of the slow migrating band corresponding to large genomic fragments. Owing to the small amount of digested DNA, you do not expect to see the smear that would correspond to cut genomic DNA fragments.

11. The ligation mix can be kept frozen at –20°C after ligation.

12. The smallest specific bands are 250 and 160 bp long when using *Mbo*I and *Mse*I, respectively.

13. It is not rare to generate several bands of different sizes from a single insertion (partial digestion of the DNA, religation of degraded DNA fragments, illegitimate PCR priming, etc.). If there are clear abundant products together with some minor products, we usually sequence only the brightest bands.

14. Sequence quality is poor if there is not enough material recovered from the gel or if there is a mix of different bands that cannot be separated on the gel. If there is not enough material, it is possible to reamplify gel-purified bands using the same primers as in **Subheading 3.4.**, **step 7**. If there is a mixture of different products, gel-purified fragments can be TA-cloned and reamplified the next day by performing PCR directly on bacteria colonies. The quality of the sequence obtained from these products is usually excellent.

Acknowledgments

References

1. Brenner, S. (1974) The genetics of *Caenorhabditis elegans*. *Genetics* **77,** 71–94.
2. Jorgensen, E. M. and Mango, S. E. (2002) The art and design of genetic screens: *Caenorhabditis elegans*. *Nat. Rev. Genet.* **3,** 356–369.
3. Wicks, S. R., Yeh, R. T., Gish, W. R., Waterston, R. H., and Plasterk, R. H. (2001) Rapid gene mapping in *Caenorhabditis elegans* using a high density polymorphism map. *Nat. Genet.* **28,** 160–164.
4. Hodgkin, J. and Doniach, T. (1997) Natural variation and copulatory plug formation in *Caenorhabditis elegans*. *Genetics* **146,** 149–164.
5. Greenwald, I. (1985) lin-12, a nematode homeotic gene, is homologous to a set of mammalian proteins that includes epidermal growth factor. *Cell* **43,** 583–590.
6. Moerman, D. G., Benian, G. M., and Waterston, R. H. (1986) Molecular cloning of the muscle gene unc-22 in *Caenorhabditis elegans* by Tc1 transposon tagging. *Proc. Natl. Acad. Sci. USA* **83,** 2579–2583.
7. Anderson, P. (1995) Mutagenesis. *Methods Cell Biol.* **48,** 31–58.
8. Plasterk, R. H. A. and van Luenen, H. G. A. M. (1997) Transposons. In: C. elegans *II* (Riddle, D. L., Blumenthal, T., Meyer, B. J., and Priess, J. R., eds.), Cold Spring Harbor Laboratory Press, New York, NY, pp. 97–116.
9. Collins, J., Saari, B., and Anderson, P. (1987) Activation of a transposable element in the germ line but not the soma of *Caenorhabditis elegans*. *Nature* **328,** 726–728.
10. Ketting, R. F., Haverkamp, T. H., van Luenen, H. G., and Plasterk, R. H. (1999) Mut-7 of *C. elegans*, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. *Cell* **99,** 133–141.
11. Tabara, H., Sarkissian, M., Kelly, W. G., et al. (1999) The *rde-1* gene, RNA interference, and transposon silencing in *C. elegans*. *Cell* **99,** 123–132.
12. Rushforth, A. M., Saari, B., and Anderson, P. (1993) Site-selected insertion of the transposon *Tc1* into a *Caenorhabditis elegans* myosin light chain gene. *Mol. Cell. Biol.* **13,** 902–910.
13. Rushforth, A. M. and Anderson, P. (1996) Splicing removes the *Caenorhabditis elegans* transposon *Tc1* from most mutant pre-mRNAs. *Mol. Cell. Biol.* **16,** 422–429.
14. Bessereau, J. L., Wright, A., Williams, D. C., Schuske, K., Davis, M. W., and Jorgensen, E. M. (2001) Mobilization of a *Drosophila* transposon in the *Caenorhabditis elegans* germ line. *Nature* **413,** 70–74.

15. Williams, D. C., Boulin, T., Ruaud, A. F., Jorgensen, E. M., and Bessereau, J. L. (2005) Characterization of *Mos1*-nediated mutagenesis in *Caenorhabditis elegans*: a method for the rapid identification of mutated genes. *Genetics* **169,** 1779–1785.

16. Jacobson, J. W., Medhora, M. M., and Hartl, D. L. (1986) Molecular structure of a somatically unstable transposable element in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **83,** 8684–8688.

17. van Luenen, H. G., Colloms, S. D., and Plasterk, R. H. (1994) The mechanism of transposition of *Tc3* in *C. elegans*. *Cell* **79,** 293–301.

18. Dawson, A. and Finnegan, D. J. (2003) Excision of the *Drosophila* mariner transposon *Mos1*. Comparison with bacterial transposition and V(D)J recombination. *Mol. Cell.* **11,** 225–235.

6

Single-Nucleotide Polymorphism Mapping

M. Wayne Davis and Marc Hammarlund

Summary

Single-nucleotide polymorphism (SNP) mapping is the easiest and most reliable way to map genes in *Caenorhabditis elegans*. SNPs are extremely dense and usually have no associated phenotype, making them ideal markers for mapping. SNP mapping has three steps. First, recombinant mutant animals are generated over a polymorphic strain (usually CB4856) using standard genetic techniques. Second, the genotype of these animals at SNP loci is determined using one of a variety of SNP detection technologies. Third, linkage between the mutant and one or more SNPs is used to position the mutant on the chromosome relative to the SNPs. This chapter presents a detailed procedure for generating recombinant animals, for assaying SNPs using restriction enzymes, and for analyzing mapping data.

Key Words: *C. elegans*; single-nucleotide polymorphism; mapping; restriction fragment polymorphism; genetics.

1. Introduction

Single-nucleotide polymorphism (SNP) mapping in *Caenorhabditis elegans* is a powerful and elegant tool for determining the chromosomal position of a mutant gene. SNP mapping as a method to clone a gene in *C. elegans* was first used by Jakubowski and Kornfeld (*1*). More recently, SNP mapping against the polymorphic strain CB4856, a strain isolated in Hawaii (*2*), was introduced by Wicks et al. (*3*), who identified a large number of polymorphisms and described the bulk segregant analysis procedure presented in this chapter. Additional polymorphisms in CB4856 were identified by the Waterston group at St. Louis, and by Exelixis (*4*). Refinements to SNP mapping techniques were developed by Exelixis (*4*), Zipperlenet al. (*5*), and Davis et al. (*6*). These advances have made SNP mapping a robust and rapid method to identify and clone a gene of interest.

SNPs have two advantages over conventional marker mutations. First, unlike conventional visible markers, SNPs in general have no phenotype, allowing a mutation of interest to be scored in a neutral phenotypic background. As a result, many markers can be assayed simultaneously, without worrying about genetic interactions. Second, SNPs between N2 and CB4856 are very dense. The average density is approximately one per kilobase of DNA *(3)*, and to date more than 15,000 SNPs have been identified. Thus, SNP mapping can provide data on very small intervals, and in some cases is capable of mapping to single-gene resolution.

Most SNP mapping experiments begin with chromosome mapping (also known as bulk segregant analysis), and then employ successive rounds of interval mapping until a narrow physical region is identified. SNP mapping can easily and quickly narrow the physical location of a gene to a very narrow region at which point cosmid rescue or sequencing experiments can be done to specifically identify the gene of interest. Both chromosome and interval mapping begin with collecting recombinants over CB4856. In chromosome mapping these recombinants are pooled, so that many recombinations are assayed at once. This allows the proportion of recombination between the allele of interest and individual SNPs to be determined. Chromosome mapping is thus a statistical assay that generally defines the chromosome and approximate position on the chromosome (left arm, middle, right arm) of a gene. Interval mapping, in contrast, involves the analysis of individual recombinants and places physical limits on the position of the allele of interest. Because of the high density of SNPs, analysis of enough recombinants will limit the position of the gene of interest to a very small interval. This chapter provides detailed protocols for chromosome and interval mapping.

SNP mapping presents two major challenges. The first, collecting an adequate number of recombinants, is not unique to SNP mapping. However, the requirement for generating recombinants over CB4856 makes this process more difficult. We discuss several solutions to this problem. Second, because of their silent nature, SNPs cannot be assayed directly. We provide a detailed protocol for detecting SNPs by restriction digest, as introduced by Wicks et al. *(3),* and discuss some alternatives to this approach.

When contemplating SNP mapping, three other techniques should be considered. First, mobile DNA elements, or transposons, can be used as mutagens *(7)*. Because insertion of an exogenous transposon can simultaneously mutate a gene and tag it with a unique molecular sequence, genes identified in forward transposon screens can be cloned without mapping. Second, the development of RNAi feeding libraries allows whole-genome screens to be performed *(8)*. This method eliminates all mapping because the molecular target of each RNAi construct is known. Likewise, when mutations are created by reverse genetic techniques, mapping is not necessary *(9)*.

However, chemical mutagenesis followed by mapping and cloning is still the preferred approach in many circumstances *(10)*. For example, many genes are resistant to RNAi, and cannot be identified in RNAi screens. Chemical mutagenesis is also the best way to generate an allelic series or to find unusual alleles. Finally, chemical mutagenesis is by far the easiest way of producing mutants, and, therefore, lends itself to complex screens. Fortunately, SNP mapping means that interesting alleles, even those with subtle phenotypes, can be quickly and accurately mapped.

2. Materials

2.1. Tools

1. 0.2 µL 96-pin replicator (hedgehog; Boekel).
2. Gel box (Owl Scientific Centipede).
3. Eight-channel pipet (20 µL vol): Rainin LTS (*see* **Note 1**).
4. Repeating pipet (optional) (Gilson Distriman).
5. 96-Well format thermal cycler (any supplier). Protocols in this chapter have been performed on MJ Research DNA Engine™ thermal cyclers.

2.2. Reagents

1. Single worm lysis buffer (SWLB) + proteinase K: 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2.5 mM MgCl$_2$, 0.45% IGEPAL CA-630 (or NP40), 0.45% Tween-20, 0.01% (w/v) gelatin. Autoclave, then store in aliquots at –20°C. Add fresh proteinase K to 60 µg/mL just before use.
2. 4X SWLB + proteinase K: 200 mM KCl, 40 mM Tris-HCl, pH 8.3, 10 mM MgCl$_2$, 1.8% IGEPAL CA-630 (or NP40), 1.8% Tween-20, 0.04% (w/v) gelatin. Autoclave, then store in aliquots at –20°C. Add fresh proteinase K to 240 µg/mL just before use.
3. 10X PCR buffer: 22.5 mM MgCl$_2$, 500 mM Tris-HCl, 140 mM (NH$_4$)$_2$SO$_4$, pH 9.2 at 25°C.
4. Restriction enzyme/restriction buffer (any supplier). Supplier lists are available at http://rebase.neb.com/rebase/rebase.html.
5. Taq DNA polymerase (any supplier).
6. dNTPs (any supplier). 10 mM each dNTP (40 mM total concentration of dNTP) in 50% sterile glycerol. This can be made from 100 mM stock solutions as: 10 µL dATP, 10 µL dCTP, 10 µL dGTP, 10 µL dTTP, 60 µL 80% glycerol (*see* **Note 2**).
7. PCR primers (any supplier). Protocols in this chapter have been preformed using primers from Integrated DNA Technologies (*see* **Note 3**).
8. Gel-loading buffer: 0.2% orange G, 0.05% xylene cyanol FF, 60% glycerol, 60 mM EDTA (*see* **Note 4**).

2.3. Disposables

1. 96-Well PCR plates (BD Falcon, cat. no. 352133).
2. PCR plate sealing mats (Genemate, cat. no. T-3161-1; distributed by ISC-BioExpress).

3. Multichannel reagent reservoir (Genemate, cat. no. B-0812-1).
4. Foil covers for PCR plates (BD Falcon, cat. no. 352143).
5. 96-Well round-bottom storage plates (BD Falcon, cat. no. 351190).
6. Lids for storage plates (BD Falcon, cat. no. 351192).

3. Methods

We provide a detailed method for chromosome mapping in **Subheading 3.1.**, and for interval mapping in **Subheading 3.2.**, using restriction-length polymorphism detection to assay SNP genotypes. These methods are also described in Davis et al. (*6*). Using these methods, a mutant gene can be mapped to an interval of 5–10 map units. To map more finely requires the design of additional SNP primers and the collection of many recombinant animals; brief protocols for these steps are given in **Subheadings 3.2.5.** and **3.2.6.** You may also want to consider using one of the other SNP detection techniques that have been employed in *C. elegans* (**Subheading 3.3.** contains a description of these). Finally, **Subheading 3.4.** discusses the mapping of challenging genotypes.

3.1. Chromosome Mapping

The first step in mapping a mutant gene is to assign the gene to a broad region of one chromosome. The method presented here uses 48 SNPs evenly spaced along the chromosomes, allowing the whole genome to be scanned simultaneously. The 48 primer sets are designed to amplify under identical conditions, and the SNPs are detected using identical restriction digestion conditions. The primers are arrayed in a 96-well plate to maximize reproducibility and to minimize set-up time. Two DNA templates are generated, one from a mixture of 50 homozygous mutant animals and a control template from nonmutant animals (or an equal mixture of N2 and CB4856 animals). Unlinked SNPs should have equal N2 and CB4856 content. Thus, mutant templates and control templates should produce an equal proportion of N2 and CB products at unlinked loci. Linkage to a SNP will result in an increase in the ratio of N2 DNA/CB DNA in the mutant relative to the control. The final output of this method is an agarose gel that has mutant and control PCR products side by side, making the differences between the mutant and the control clearly visible.

3.1.1. Genetics

1. Obtain homozygous CB4856 males (*see* **Note 5**).
2. Cross them into homozygous *mut/mut* hermaphrodites (*see* **Note 6**).
3. After 24 h the hermaphrodites should have a mating plug. Clone each mated hermaphrodite to a fresh plate (*see* **Note 7**).
4. In 3 d clone 8–12 L4 hermaphrodites; these should be *mut*/CB4856 (*see* **Note 8**).

5. Wait 3–4 d, or until Mut and non-Mut progeny can be distinguished (*see* **Note 9**).
6. Put 50 Mut animals into a tube containing 20 μL SWLB + proteinase K. Put 50 non-Mut animals in a second tube (*see* **Note 10**).
7. Freeze tubes at –80°C for at least 10 min. This is a convenient place to stop if so desired.

3.1.2. Chromosome Mapping Primers

Primer pairs are premixed in a separate 96-well plate and added to the reaction by pin replication. This reduces both reaction preparation time and the potential for errors. The primers are formatted to produce a gel that has each mutant digest next to its control, moving in order from LG IL to LG XR. To accomplish this, the primers are set up in a 96-well format so that row 1 contains the eight primer pairs for LG I, with well 1H containing primers for the left-most marker. Row 2 is a duplicate of row 1. Rows 3 and 4 are duplicates and contain the LG II primer pairs, rows 5 and 6 have primers for LG III, and so on. When the reactions are loaded onto the gel using a multichannel pipet duplicate rows will be interleaved, placing each mutant reaction directly adjacent to its corresponding control.

Dissolve primers in 10 m*M* Tris-HCl, pH 8.0, and add primers to each well of the round-bottomed storage plates at 10 μ*M* each primer. The primer stock plates can be frozen in a nonfrost-free freezer and reused many times; however, multiple plates should be made at the same time with only 20 μL per well because plates can go bad for a number of reasons (left out of freezer, hot pin replicator, cross-contamination, dropped, and so on). The plates should always be briefly spun in a tabletop centrifuge before removing the lids in order to prevent cross-contamination owing to condensation in the lid.

3.1.3. SNP Genotyping

1. Lyse worms. Incubate 65°C for 1 h, 95°C for 15 min. After lysis, keep template on ice and store at –80°C when not in use to prevent degradation of DNA.
2. Set up PCR master mixes. For each mutant you are mapping, make two tubes of the following: 52 μL 10X PCR buffer, 10.4 μL 10 m*M* dNTPs, 3.12 μL Taq (5 U/ μL), 424 μL water. Add 20 μL of your Mut lysis to one tube, and 20 μL of your non-Mut lysis (or control DNA) to the other. Mix thoroughly.
3. Add master mixes to the plate. Using a multichannel or repeating pipet, aliquot 9.8 μL of the Mut mix into rows 1, 3, 5, 7, 9, and 11 of a 96-well PCR plate. Aliquot 9.8 μL of the non-Mut (or control) mix into rows 2, 4, 6, 8, 10, and 12.
4. Pin replicate primers. Dip replicator into primer plate and then into PCR plate (*see* **Note 11**).
5. PCR. Cycling parameters: 94°C for 2 min, (94°C for 15 s, 60°C for 45 s, 72°C for 1 min) 35 times, 72°C for 5 min. Because the volumes are small, a heated lid is critical to prevent evaporation.

6. Digest. Mix 168 μL 10X *Dra*I buffer, 26.25 μL (262 U) *Dra*I, 435.75 μL water. Aliquot 6 μL into each PCR well, and centrifuge the plate to mix. Incubate at 37°C for 4 h overnight. Evaporation during this step can be a problem. Incubation is best accomplished in a thermocycler with a heated lid. Alternatively, plates can be sealed carefully with aluminum sealing film, making sure each well is fully sealed, followed by incubation in a 37°C air incubator.
7. Add gel-loading buffer. Using a multichannel or repeating pipet add 4 μL of loading buffer to each well (*see* **Note 4**).
8. Run the gel. Pour a 2.5% agarose gel using TAE or TBE and put two 50-well combs into the tray. When the gel is ready, load the gel using an eight-channel pipettor. Load row 1 (1H–1A), starting in the first well of the 50-well comb. Load row 2 (2H–2A) interleaved with row 1, starting in the second well. Leave a space, and load rows 3 and 4 interleaved, followed by another space and then rows 5 and 6.
9. This should fill the 50 wells. Load the bottom lanes with rows 7–12. Put 50 bp ladder in the four empty spaces.

3.1.4. Analyzing SNP Data

Unlinked SNPs should have equal N2 and CB4856 content and thus, mutant templates and control templates should produce an equal ratio of cut-to-uncut products. Linkage to a SNP is determined by an increase in the ratio of N2 DNA to CB4856 DNA in the mutant product relative to the control. Depending on the SNP, this may be an increase in cut or in uncut DNA. The ratio is the relevant parameter, not the absolute amount of product. When using eight SNPs per chromosome, a mutation will show tight linkage to at least one SNP and noticeable linkage to adjacent SNPs. Weak linkage to one SNP, but not to adjacent SNPs, is almost always a spurious result.

3.2. Interval Mapping

Once your mutation has been assigned to a broad region of a chromosome using chromosome mapping, interval mapping can be used to narrow the interval to a region small enough for molecular cloning techniques to be applied. Briefly, individual recombinant animals are genotyped for SNPs in the region to which the gene has been mapped. The closest break point on either side of the mutation then defines the physical limit of the mutation's position. To reduce the size of this interval, additional SNPs within the interval can be assayed. Only those animals that are recombinant within the interval (i.e., have different SNP genotypes at the ends of the interval) are likely to provide additional information. Once the limits are moved so close that there are no more informative recombinants, additional recombinants must be collected, as described in **Subheading 3.2.6.**

3.2.1. Genetics

1. Generate recombinants. Follow **steps 1–5** of **Subheading 3.1.1.** These animals can be generated from the same crosses used for chromosome mapping (*see* **Note 10**).

2. Single at least 200 homozygous Mut hermaphrodites onto individual seeded plates.
3. Take some time to examine the self-progeny brood and verify that each plate is indeed homozygous mutant. Discard any plates that appear questionable.
4. Label the plates clearly, corresponding to a 96-well plate format.
5. Just before the plates starve, add 250 μL sterile water to each plate, tip the plate, and transfer 100 μL containing worms to the well of a 96-well plate. The amount of water can be adjusted based on the size of the plate used and the dryness of the agar. It is recommended to work in sets of four, six, or eight plates at a time. Make sure that the well and the plate label correspond. Use a new pipet tip for each plate to avoid cross-contaminating plates or wells. Also make a well with N2, a well with CB4856, and a well with an equal mix (*see* **Note 12**).
6. Store the starved plates at 15°C for later reference.
7. Place the 96-well plates at 4°C for 15 min to overnight to allow the worms to settle.
8. Check all of the wells to verify that they have approximately equal volumes and a clearly visible pellet of worms.
9. Carefully remove 55 μL from the top of each well, leaving the worm pellet undisturbed. The remaining volume should be 45 μL.
10. Add 15 μL 4X SWLB + proteinase K to each well.
11. Freeze at –80°C with a foil lid. This is a convenient place to stop. However, freeze even if proceeding immediately, as it helps with lysis.

3.2.2. SNP Genotyping

Inspect your chromosome mapping results and select the three to five adjacent markers that are most tightly linked to your mutation. You will test each of your recombinants for these markers and generate a snapshot of the recombinant chromosomes in the region of your mutant. Make sure the plates are well labeled, including registration marks indicating the orientation of the plate.

1. Lyse worms. Mix the settled worm pellet and SWLB by vortexing the 96-well plate. Incubate at 65°C in a thermocycler with a heated lid for 30 min. Vortex again. Incubate at 65°C for 30 min, then heat inactivate the proteinase by heating to 95°C for 15 min. After lysis, keep template on ice and store at –80°C when not in use to prevent degradation of DNA.
2. Make a separate master mix for each SNP you wish to genotype. The mixes will differ only in their primers. The volume of mix depends on the number of SNPs and the number of recombinants. *See* **Table 1** for volumes. Add 9.8 μL master mix to each well of the plates.
3. Add templates to the 96-well plates by pin replication (*see* **Note 11**). More reliable results have been obtained when the pin replication process is repeated a second time.
4. PCR. Use the same conditions as described in **Subheading 3.1.3.**, or conditions empirically optimized for each new primer set.
5. Digest. Add 6 μL restriction digestion mix to each well. *See* **Table 2** for volumes.
6. Run TAE agarose gel as described in **Subheading 3.1.3.**

Table 1
Interval Mapping PCR Mix Volumes

	For each 10 μL rxn	1 plate	2 plates	3 plates	4 plates
H_2O	8.50 μL	850 μL	1700 μL	2550 μL	3400 μL
10X PCR buffer	1.00 μL	100 μL	200 μL	300 μL	400 μL
10 mM dNTP	0.20 μL	20 μL	40 μL	60 μL	80 μL
Taq polymerase	0.06 μL	6 μL	12 μL	18 μL	24 μL
Fwd primer 100 μM	0.02 μL	2 μL	4 μL	6 μL	8 μL
Rev primer 100 μM	0.02 μL	2 μL	4 μL	6 μL	8 μL
Total	9.80 μL	981 μL	1962 μL	2943 μL	3924 μL

Table 2
Interval Mapping Digest Mix Volumes

	For digesting each 10 μL PCR	1 plate	2 plates	3 plates	4 plates
H_2O	4.15 μL	415 μL	830 μL	1245 μL	1660 μL
10X buffer	1.60 μL	160 μL	320 μL	480 μL	640 μL
Enzyme	0.25 μL	25 μL	50 μL	75 μL	100 μL
Total	6.00 μL	600 μL	1200 μL	1800 μL	2400 μL

3.2.3. Analyzing SNP Data

We have found that the easiest way to analyze interval mapping SNP data is to put the data into a simple spreadsheet, with a row for each singled plate (the F_2 generation) and a column for each SNP. It is helpful to color each cell depending on the genotype (N2, CB4856, or heterozygote) by using the conditional formatting function in Excel. If you have selected SNPs that are close to your mutant, most animals will be homozygous N2 at all SNPs. Some animals will carry recombinant chromosomes and be heterozygous at one or more adjacent SNPs. These recombinant animals provide information about the position of the mutation, because the mutation cannot be in the region that is heterozygous. By comparing all the recombinants, the minimal interval containing the mutation can be identified.

The key is to find two SNPs that are near your mutation, but flank it. At this point, for further mapping experiments you need only genotype each animal for the two nearest flanking SNPs. Animals homozygous N2 at both of these SNPs are uninformative and need not be assayed further. This will immediately cut your large number of SNP assays to a small number of informative

recombinant DNA templates. A sample that is heterozygous at the flanking SNP should be reassayed for intermediate SNPs. As you assay these different SNPs, fewer samples will be heterozygous, until you have found two SNPs that each have a single heterozygote. These SNPs mark the left and right boundaries of the genetic interval that contains your gene of interest. If the boundary SNPs are sufficiently close to each other or bracket a sufficiently promising candidate gene, other molecular techniques can be used to clone the gene (transgene rescue, sequencing). If they are too far apart, a new collection of recombinants should be collected and assayed for the SNPs that mark the left and right of your interval.

In some cases, the problem arises that the initial set of SNPs are all on one side of your mutation. In this case, the problem can be solved by assaying new SNPs until you have found an SNP on the other side. Another common problem is the occurrence of rare genotypes that are inconsistent with a unique map position. The genotypes may be because of misscoring the phenotype. When the plates corresponding to these SNP reactions are regrown and re-examined for the phenotype it is almost always discovered that they have indeed been misscored. Finally, it is possible to see no obvious linkage from the genotypes. In these cases, it may be that the chromosome mapping gel has been misinterpreted. It is also possible that the phenotypes that were selected are actually represented by multiple genotypes.

3.2.4. Designing New SNP Primers

When using SNP mapping to clone a gene, you will almost always have to go through several rounds of designing SNP detection primers that provide information in subsequently narrower genetic intervals. There are several web-based tools that assist worm researchers in identifying verified and potential SNPs that have been found using shotgun sequencing of CB4856. Most of this data has been provided by two groups: one at the Genome Sequencing Center at Washington University at St. Louis *(3)*, and one at Exelixis *(4)*. They have both made their primary data available on the web. The St. Louis data are available at website: http://genome.wustl.edu/projects/celegans/index.php?snp=1 and the Exelixis data are available at website: http://www.exelixis.com/index.asp?secPage=elegans. These data have been combined and made searchable at the WormBase website (http://www.wormbase.org/db/searches/strains). This website is geared towards mapping against visible markers but can provide SNP data in an interval. It can filter the SNPs for verified or unverified and for snip-SNP (contains a polymorphism that changes a restriction site) or nonsnip-SNP (contains a polymorphism that can only be identified by sequence). For verified SNPs, the primers used by the verifying lab are given. At the time of writing, the site was still very much a work in progress, with inconsistent results that fre-

quently listed snip-SNPs as only detectable by sequencing, and listing numerous unavailable restriction enzymes.

Alternatively, a Filemaker (Filemaker Inc., http://www.filemaker.com) database file that contains data from both sequencing groups analyzed for polymorphic restriction sites has been compiled by the authors and is available from http://www.biology.utah.edu/jorgensen/SNPs.

When a promising SNP has been identified, primers that amplify the SNP fragment must be designed. A wide variety of primer design methods and utilities have been described, but the authors have found that the web-based primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) produces reliable results. Exact primer properties seem to be unimportant in achieving SNP results, but designing all SNP primers with a uniform melting temperature can often assist in minimizing PCR optimization steps and often allows multiple SNPs to be assayed in a single batch of PCR. Obviously, primers should be designed to provide a detectable difference in restriction fragment length. We have found that using 2.5% agarose gels bands with 30 bp differences can be detected, with 50 bp the smallest absolute size. If possible, the two bands produced by restriction digestion should be of different sizes. In addition, some people prefer to have a nonpolymorphic site present in the PCR product as a positive control for digestion. However, a nonpolymorphic site too close to the SNP site will make the SNP impossible to detect by restriction digestion.

Even if you chose to use primers previously designed and tested by others, it is a good idea to test the primers on at least N2, CB4856, and a mixture of N2 and CB4856 DNA before embarking on a massive PCR screen. If time and equipment provide, a gradient PCR machine should be used to empirically find an optimal annealing temperature for each new primer set.

In some cases, the known SNPs will not be sufficiently dense to provide satisfactory map information for cloning a gene. In this case, the researcher will have no other option but to look for SNPs of their own, by direct sequencing of CB4856. The density of known SNPs suggests that an SNP can be found in approximately every 1 kb (*3*). The actual SNP frequency may be very different in different regions of the genome, but a few sequencing reads from PCR products concentrated in noncoding regions should provide a SNP. Approximately 50% of new SNPs should be snip-SNPs, and 60% or more of these can produce restriction fragments with large enough size differences for detection on agarose gels (*3*).

3.2.5. Directed Mapping

It is an unfortunate truth that the closer one gets to one's gene, the more difficult mapping becomes. This is a simple consequence of informative recombinants comprising a smaller and smaller fraction of the whole. For example, if

in the first round of interval mapping you position your gene between two SNPs that are five map units apart, you can expect that only 10% of future recombinants will fall in that interval and, thus, provide information. In many cases, the application of brute force will solve this problem by collecting many recombinants, genotyping them at the flanking SNP loci, and saving for further analysis those animals that have different genotypes at these loci. These are recombinant in the region of interest, and can be analyzed further.

However, a more elegant solution also exists. This solution relies on using recombination between visible markers to enrich for recombination events in the region of interest. We describe next three experimental approaches for directed mapping. Only the genetics part of SNP mapping is covered. Once recombinants have been collected, the mapper should assay their SNP genotypes using the methods described in **Subheading 3.2.2.**

3.2.5.1. METHOD 1: TWO-FACTOR MAPPING

You have established that your gene is flanked by SNP *a* and SNP *b*, and you wish to enrich for recombinants within the *ab* region. To use a two-factor approach, you need to construct a double mutant containing your gene (*mut-1*) and a visible marker (*vis-1*). The marker should lie outside but close to the *ab* interval, and you will obtain map data only on the side the marker is on. Also, you must be able to reliably distinguish at least one of the recombinant phenotypes (Mutant non-Marker or Marker non-Mutant) from the parental phenotypes (Mutant Marker and wild-type).

Hermaphrodites with the genotype *vis-1 mut-1/CB4856* are allowed to self. These heterozygotes will segregate rare progeny with the recombinant phenotypes Mutant non-Marker and Marker non-Mutant. These animals will have at least one chromosome that is recombinant in the *vis-1-mut-1* interval. If *vis-1* is close to SNP *a,* many of these crossovers will be in the *a-mut-1* interval and thus informative about the limit of *mut-1* on the side containing SNP *a*.

This approach can be useful if there are good visible markers near your SNP boundaries. Because it only generates data on one side, two parallel experiments are usually necessary.

3.2.5.2. METHOD 2: THREE-FACTOR MAPPING

Three-factor mapping has two large advantages over two-factor mapping. First, information is obtained on both sides of the mutation from a single experiment. Second, it is not necessary to score the mutant phenotype when collecting recombinants. The initial procedure is similar to two-factor mapping, except the mutant is marked on both sides. To use a three-factor approach, you need to construct a triple mutant containing your gene and two visible markers, *vis-1* and *vis-2*. The markers should flank the *ab* interval as closely as possible. You should

be able to reliably distinguish at least one of the recombinant marker phenotypes (Marker 1, non-Marker 2, or Marker 2, non-Marker 1) from the parental phenotypes (Marker 1, Marker 2, and wild type).

Hermaphrodites with the genotype *vis-1 mut-1 vis-2/CB4856* are allowed to self. These heterozygotes will segregate rare progeny with the recombinant phenotypes Marker 1, non-Marker 2, or Marker 2, non-Marker 1. These animals will have at least one chromosome that is recombinant in the *vis-1-vis-2* interval. These animals can be scored for *mut-1* immediately, or cloned and scored in the next generation as a population.

Again, this approach requires good visible markers, and also requires the construction of a fairly complex strain. If these conditions are met, this approach is very powerful.

3.2.5.3. METHOD 3: USING THE CB4856 BACKGROUND

In this approach, mutations are generated in the CB4856 background and mapped against N2, rather than the reverse. Either two- or three-factor mapping can then be done, with the significant advantage of not having to mark the mutant strain. Instead, the mutant is simply placed over a marked N2 strain. Recombination either between the mutation and an N2 marker or between two N2 markers can be used to enrich for informative recombinants.

This approach is attractive because it does not require strain building. There are many existing double-marker strains in the N2 background, in lab collections or available from the CGC. However, having the mutation of interest in the CB4856 background could prove problematic for later study.

3.3. SNP Detection Technology

Several SNP detection techniques have been shown to work well in *C. elegans*. The first technique described for SNP detection in *C. elegans*, and still the most widely used, is restriction digestion detection after PCR *(3)*—the strategy used in the previously mentioned protocols for chromosome and interval mapping. In 2002, Swan et al. *(4)* published an application of fluorescence polararization-template directed incorporation (FP-TDI) for use in SNP detection in *C. elegans*. More recently, a method involving detection of small insertion/deletion (InDel) polymorphisms using capillary sequencing has been developed by Zipperlen et al. *(5)*. Many other techniques, including oligonucleotide microarray hybridization *(11)*, locked nucleic acid (LNA) hybridization *(12)*, or allele-specific extension *(13)*, rolling-circle amplification *(14)*, Taqman *(15)*, and mass spectroscopy *(16)*, are used to detect SNPs in other systems and could be applied to *C. elegans* after investment in development of the necessary worm-specific reagents. The choice of a particular technique for SNP detection will ultimately depend on consideration of your specific requirements and resources.

3.3.1 Restriction Fragment-Length Polymorphism Detection

Restriction digestion SNP (known as snip-SNP) detection is currently the most commonly used technique in the worm field, probably because it has low equipment and expertise requirements. Because it depends on the SNP changing a recognition site for a restriction enzyme, a major limitation of this technique is that not all SNPs can be detected. However, snip-SNPs between N2 and Hawaiian are dense enough to give resolution finer than one map unit in most genomic regions. This is usually dense enough to allow cloning the gene, or to limit the number of animals bearing informative recombinant chromosomes to a small enough number that additional SNPs can be assayed by DNA sequencing. Another drawback of snip-SNP detection is that each SNP in a genomic region usually requires a different restriction enzyme. This means that frequently each SNP requires different digestion conditions. This adds additional hands-on processing time and becomes a potential source of confusion if multiple SNPs are assayed at the same time. Further, some SNPs can only be detected with relatively expensive or difficult to obtain enzymes.

3.3.2. FP-TDI Detection

Detection of SNPs by FP-TDI relies on the fact that a fluorescently labeled nucleotide in solution has different fluorescence polarization characteristics than one incorporated in a DNA strand. Dye terminator nucleotides are used in a primer extension reaction at the SNP, and fluorescent polarization information is used to detect which base has been added and thus identify the SNP genotype. FP-TDI overcomes some of the limitations of snip-SNP detection, but requires an initial investment in a moderately expensive piece of equipment (a plate-reading fluorescence polarimeter). Unlike many other methods, FP-TDI can be used to detect any sequence polymorphism. In addition to many confirmed *C. elegans* FP-TDI primers identified by Exelixis, many *C. briggsae* FP-TDI primers have been identified by Washington University. These primers are available at http://snp.wustl.edu/snp-research/c-briggsae/. Because all of the detection reactions can be carried out in a single well of a 96- or 384-well plate, FP-TDI can be automated, reducing the hands-on time and the potential for mistakes. Unlike snip-SNP detection, no SNP-specific enzymes are required for FP-TDI. However, because of the expense of some of the reagents used in FP-TDI the cost per reaction is comparable or higher than snip-SNP detection. In addition, the plate reading fluorescence polarimeter costs tens of thousands of dollars.

3.3.3. InDel Detection

The InDel detection technique described by Zipperlen et al. *(5)* has a mix of advantages and disadvantages over snip-SNP detection and FP-TDI detection.

Like snip-SNP detection, InDel detection is limited to a subset of SNPs. In this case, it is limited to SNPs that insert or delete one or two base pairs, estimated to be about 20% of all *C. elegans* SNPs *(5)*. As in the other techniques, PCR is used to amplify the region containing the polymorphism. However, in this technique one of the primers must be fluorescently labeled. The fluorescent PCR product is then run on an ABI capillary sequencer to resolve the PCR products that vary by 1 or 2 bp. This approach has the advantages of automation found in FP-TDI and simplicity of snip-SNP detection. However, the availability of the appropriate equipment and the cost of reagents may limit the access of some labs to this technique. A variation of this technique, called SNPwave, which uses an initial ligation mediated detection step to detect single base pair changes, has been published by van Eijk et al. *(17)*.

3.4. Mapping Challenges

3.4.1. Complex Genotypes

Previous sections discussed mapping of simple recessive loss of function alleles. However, an added benefit of SNP mapping is that genes contributing to complex phenotypes can also be mapped relatively easily. For example, semidominant or dominant alleles, phenotypes that require mutant alleles at more than one locus, and second-site enhancer and suppressor alleles can all be mapped using SNPs.

The key to SNP mapping these complex genotypes is to identify a phenotype segregating from a heterozygous animal that results from a single, unique genotype. This will almost always be a homozygous genotype. In the simple case of a single recessive allele, the Mut phenotype results only from the homozygous *m/m* genotype. However, for dominant alleles, the Mut phenotype is found in both the heterozygote and homozygous mutant genotypes. Thus, in the case of a dominant mutation, the phenotype that should be selected is wild-type, because this results only from the homozygous *+/+* genotype. It may seem counterintuitive to go though the entire cross and then pick entirely wild-type animals, but this is the best way to obtain unambiguous mapping results with dominant alleles. Because the wild-type allele is present in the Hawaiian genome, linkage will be detected as an increase in Hawaiian DNA (the opposite of mapping a recessive allele).

In the case of some allelic interactions, the first generation of animals segregating from a heterozygote (the F_2 generation) will not contain a phenotype associated with a unique genotype. For example, this is the case for a recessive suppressor of a recessive mutation if the suppressed phenotype is indistinguishable from wild-type. In these cases, one can often first self F_2 Mut animals and then identify suppressed segregants in the F_3 generation, which will now have a unique homozygous suppressor genotype. In other cases of allelic interac-

tions, the double homozygote has a unique phenotype in the F_2. In this case the animals can be found in the F_2, albeit at only one-sixteenth of the population.

A different problem arises when a phenotype can only be reliably assayed as a population. A viable way to proceed in this case is to blindly single many F_2 progeny of a heterozygote and assay each of their broods. For a recessive mutation, broods that are 100% Mut should be saved and their DNA collected for SNP analysis as described in **Note 10** and in **Subheading 3.2.1.**

3.4.2. CB4856 Background Effects

It should be noted that CB4856 is not phenotypically identical to N2 in all respects. It is currently known that CB4856 has alleles of *npr-1*, *plg-1*, and *ppw-1* that are phenotypically different from N2 *(18–20)*. In some cases the differences may be detrimental to phenotyping, and thus SNP mapping, of some mutations. For example, the *npr-1* allele may interfere with many types of assays that involve observing taxis behaviors, as *npr-1* mutant animals have a strong tendency to crawl at the borders of a bacterial lawn and a strong tendency to clump together *(18)*. *ppw-1* makes CB4856 animals resistant to RNAi induced by feeding, but not by dsRNA injection *(20)*. This fact complicates mapping any phenotype that requires RNAi as part of the phenotypic assay. Likewise, the mating plug deposited by *plg-1* mutants may interfere with male mating assays. It should not simply be assumed that if your phenotype would not obviously be affected by these alleles that you are safe. CB4856 may still have an interfering effect on your phenotype. For example, CB4856 animals have an altered response to ethanol because of their modified *npr-1* allele *(21)*. Further, it remains to be seen whether there are additional CB4856 polymorphisms with phenotypic consequences, a fact which should be considered if unusual phenotypes appear to arise in your assays after outcrosses to CB4856.

4. Notes

1. It is critical that the tips align perfectly in the multichannel pipettor, a problem solved by the square geometry of the LTS design. Pipettors using conical tips have been found to perform poorly for loading gels.
2. dNTPs are very sensitive to freeze–thaw cycles. They should always be stored at –20°C in a *nonfrost-free freezer*. The glycerol in the solution prevents multiple freeze–thaw cycles from degrading the dNTPs. Alternatively, a 10 mM dNTP stock can be made in water and frozen in single use aliquots. All enzymes and dNTPs should be maintained at less than 0°C using a benchtop freezer block when in use.
3. PCR primers can be sensitive to low pH present in ddH$_2$O. Primer stocks should always be dissolved in buffered Tris-HCl, pH 8.0.
4. Any preferred gel-loading buffer can be used. The additional step of adding the gel loading buffer after digestion can be eliminated by adding 1 μL of 30% sucrose

plus 0.5 μL of 0.1% cresol red in the restriction digest mix. Although many restriction enzymes work after sucrose addition, it is not known whether all enzymes work as efficiently in this condition.

5. These animals can be difficult to recover from a mating plate because of the strain's tendency to burrow, but vortexing the plate will bring worms to the surface. Alternatively, you can remove the P_0s after 24 h of mating or use agarose plates.

6. For single matings, use 3 hermaphrodites and 10 males. Use more hermaphrodites if they are difficult to mate to, or if singling the mated animals.

7. This step enriches for cross-progeny. It is not necessary unless cross-progeny cannot be distinguished from self-progeny.

8. If you have singled mated worms, clone only from those plates that have segregated approx 50% males. It is not a bad idea to clone 8–12 additional *mut*/CB4856 animals and put them at 15°C, just in case. If you want to collect a very large number of recombinants, clone more worms.

9. Plates not segregating Mut, and plates segregating males, should be discarded. Some phenotypes are easier to score than others. If your phenotype cannot be reliably distinguished on a mixed plate, *see* **Note 10**.

10. If you cannot score your phenotype in individual worms, clone 200 self-progeny of *mut*/CB4856 hermaphrodites to individual plates. Wait until you can distinguish the homozygous mutant plates; discard the rest. For chromosome mapping, multiply the number of confirmed homozygous plates by 4 μL. Into that volume of SWLB + proteinase K pick about 10 worms from each plate. This will be your Mut lysis. For your non-Mut control, put 25 N2 and 25 CB4856 worms into a tube containing 20 μL SWLB+ proteinase K. If you can distinguish Mut from non-Mut animals, single 200 Mut F_2 animals at this point for later use in interval mapping.

11. Clean pin replicator by dipping the pins, about half way up the length of the pin, in ethanol, then flaming it. Be aware that flaming ethanol can sometimes drip off of the replicator or flare upward off of the replicator. Never put your hands, face, or hair above or below the flaming replicator. Before starting, make sure to clear your work area of flammable items like paper, especially if it is hanging from walls or shelves. When the ethanol tray catches fire, do not panic. At worst the ethanol will burn out eventually if you do not spread it around. Simply cover the tray with a nonflammable item like a plate or beaker to smother the flame. Allowing the pins to cool to room temperature is critical, because primers and templates are severely damaged by hot pins. After the ethanol has burned off, waving the replicator in a broad arc speeds the cooling—just make sure you do not drop it or hit someone behind you. Pay close attention to keeping the orientation of the plates consistent, so that you will have the same alignment in your assay plate as in the PCR primer plate. Also make sure that you are placing the pins into all of the wells; it is easy to get out of register and miss the first or last row of the plate. Rocking the replicator slightly after placing it into the sample can assure that all of the pins have hit the bottom of the wells.

12. In some cases, the plates will not all reach starvation at the same time. In addition, some plates may burrow. Some degree of judgment may be required to harvest at the perfect time to optimize the yield of worms. It is usually best to wait until at least most of the plates have almost starved, even if that means many plates have been starved for a day or so. Adequate yield can easily be obtained from recently starved plates. In the case of burrowed plates, vortexing the plate for 5–10 s will usually drive most of the worms to the surface after a few minutes, allowing worms to be washed off. Yield from contaminated plates is often low. In the case of contamination of a few of the plates with slime or mold, it is often easiest to discard the plates rather than risk spreading the contamination. Minimizing contamination also aids in the storage of the plates for later reference.

References

1. Jakubowski, J. and Kornfeld, K. (1999) A local, high-density, single-nucleotide polymorphism map used to clone *Caenorhabditis elegans cdf-1*. *Genetics* **153,** 743–752.
2. Hodgkin, J. and Doniach, T. (1997) Natural variation and copulatory plug formation in *Caenorhabditis elegans*. *Genetics* **146,** 149–164.
3. Wicks, S. R., Yeh, R. T., Gish, W. R., et al. (2001) Rapid gene mapping in *Caenorhabditis elegans* using a high density polymorphism map. *Nat. Genet*. **28,** 160–164.
4. Swan, K. A., Curtis, D. E., McKusick, K. B., et al. (2002) High-throughput gene mapping in *Caenorhabditis elegans*. *Genome Res*. **12,** 1100–1105.
5. Zipperlen, P., Nairz, K., Rimann, I., et al. (2005) A universal method for automated gene mapping. *Genome Biol*. **6,** R19.
6. Davis, M. W., Hammarlund, M., Harrach, T., et al. (2005) Rapid single nucleotide polymorphism mapping in *C. elegans*. *BMC Genomics* **12,** 118.
7. Bessereau, J. L., Wright, A., Williams, D. C., et al. (2001) Mobilization of a *Drosophila* transposon in the *Caenorhabditis elegans* germ line. *Nature* **413,** 70–74.
8. Fraser, A. G., Kamath, R. S., Zipperlen, P., et al. (2000) Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. *Nature* **408,** 325–330.
9. Zwaal, R. R., Broeks, A., van Meurs, J., et al. (1993) Target-selected gene inactivation in *Caenorhabditis elegans* by using a frozen transposon insertion mutant bank. *Proc. Natl. Acad. Sci. USA* **90,** 7431–7435.
10. Jorgensen, E. M. and Mango, S. E. (2002) The art and design of genetic screens: *Caenorhabditis elegans*. *Nat. Rev. Genet*. **3,** 356–369.
11. Sapolsky, R. J., Hsie, L., Berno, A., et al. (1999) High-throughput polymorphism screening and genotyping with high-density oligonucleotide arrays. *Genet. Anal*. **14,** 187–192.
12. Johnson, M. P., Haupt, L. M., and Griffiths, L. R. (2004) Locked nucleic acid (LNA) single nucleotide polymorphism (SNP) genotype analysis and validation using real-time PCR. *Nucleic Acids Res*. **32,** E55.

13. Latorra, D., Campbell, K., Wolter, A., et al. (2003) Enhanced allele-specific PCR discrimination in SNP genotyping using 3' locked nucleic acid (LNA) primers. *Hum. Mutat*. **22,** 79–85.

14. Qi, X., Bakht, S., Devos, K. M., et al. (2001) L-RCA (ligation-rolling circle amplification): a general method for genotyping of single nucleotide polymorphisms (SNPs). *Nucleic Acids Res*. **29,** E116.

15. Livak, K. J. (1999) Allelic discrimination using fluorogenic probes and the 5' nuclease assay. *Genet. Anal*. **14,** 143–149.

16. Ross, P., Hall, L., Smirnov, I., et al. (1998) High level multiplex genotyping by MALDI-TOF mass spectrometry. *Nat. Biotechnol*. **16,** 1347–1351.

17. van Eijk, M. J., Broekhof, J. L., van der Poel, H. J., et al. (2004) SNPWave: a flexible multiplexed SNP genotyping technology. *Nucleic Acids Res*. **32,** E47.

18. de Bono, M. and Bargmann, C. I. (1998) Natural variation in a neuropeptide Y receptor homolog modifies social behavior and food response in *C. elegans*. *Cell* **94,** 679–689.

19. Hodgkin, J. and Doniach, T. (1997) Natural variation and copulatory plug formation in *Caenorhabditis elegans*. *Genetics* **146,** 149–164.

20. Tijsterman, M., Okihara, K. L., Thijssen, K., et al. (2002) PPW-1, a PAZ/PIWI protein required for efficient germline RNAi, is defective in a natural isolate of *C. elegans*. *Curr. Biol*. **12,** 1535–1540.

21. Davies, A. G., Bettinger, J. C., Thiele, T. R., et al. (2004) Natural variation in the *npr-1* gene modifies ethanol responses of wild strains of *C. elegans*. *Neuron* **42,** 731–743.

7

Creation of Transgenic Lines
Using Microparticle Bombardment Methods

Vida Praitis

Summary

Introduction of exogenous DNA into *Caenorhabditis elegans* is important for examining the expression of altered or reporter gene constructs, rescuing mutant genes, and studying gene function in vivo. Until recently, germ-line injection was the most commonly used method for transforming *C. elegans* strains. This chapter describes four different microparticle bombardment methods used to transform *C. elegans* with exogenous DNA. We include a discussion of the advantages and disadvantages of using microparticle bombardment for transformation, list cotransformation markers that have been used successfully in microparticle bombardment experiments, and discuss transformation efficiency.

Key Words: *C. elegans*; biolistic; microparticle bombardment; gene gun; extrachromosomal arrays; integrated lines; transformation.

1. Introduction

Introduction of exogenous DNA into *Caenorhabditis elegans* has been used to express reporter genes, study gene structure and function in vivo, and to rescue mutant genes. The most commonly used method for DNA transformation has been the germ-line injection method. In this method, individual hermaphrodites are microinjected with exogenous DNA, which becomes incorporated into the genome of developing oocytes, and F1 progeny are screened for expression of the introduced genes. Typically, transformed lines produced using this method carry extrachromosomal arrays containing 80–300 copies of the exogenous DNA construct. Extrachromosomal arrays are not transmitted with 100% fidelity unless the array is incorporated into a chromosome by inducing non-homologous double-strand break repair *(1–5)*. Although the germ-line injection approach has been extremely powerful in advancing our understanding of gene

expression in *C. elegans*, it has some drawbacks. First, the approach is technically difficult, especially for new researchers, and requires expertise in setting up in a new laboratory. In addition, if the extrachromosomal arrays are not integrated, they are lost at some frequency, which can make gene rescue and expression patterns difficult to interpret. Finally, analysis of gene expression from lines made by the germ-line injection method is imperfect because the high copy number of genes carried on extrachromosomal or integrated arrays can lead to gene overexpression or silencing *(3,5–8)*. Because of the drawbacks associated with germ-line injection methods, it has been difficult for researchers to analyze genes that are dose sensitive or easily silenced when present in high copy number.

Modifications of the germ-line injection method have been used to create low copy and homologous integrated lines, which overcome many of the problems associated with extrachromosomal arrays. These modifications include addition of the *sup-7* poison sequence or oligonucleotides in the exogenous DNA and direct injection of oocytes *(3,4,9,10)*. However, the difficulty in transforming a large number of individuals required for success has limited the use of these modifications. A recently developed method for targeted gene alteration in *C. elegans* uses extrachromosomal transgenes introduced by germ-line injection as templates for gene conversion at transposon insertion sites *(11)*. Other changes to the germ-line injection method include creation of "complex" arrays that carry fewer copies of the gene of interest, which decreases chances of gene silencing. This modification has been used successfully to express genes in the germ line, where silencing is particularly acute *(12)*.

In recent years, the microparticle bombardment technique has been modified to introduce exogenous DNA into *C. elegans*. Rather than handling individuals, this approach relies on transforming a population of hermaphrodites with a spray of DNA-coated beads and selecting transformed individuals from the next generations. The technique has been used successfully to create extrachromosomal array lines, low-copy integrated lines, and homologous recombinants of genes that are expressed in both the soma and germ line *(13–16)*. The microparticle bombardment methods for creating extrachromosomal transgenes should also be compatible with the new method to direct gene conversion events at transposon insertions sites *(11)*. A microparticle bombardment approach has been used as a way to introduce dsRNA hairpin constructs (hpRNAi) to target tissues refractory to other RNAi delivery methods; for example *unc-4,* a gene highly resistant to conventional RNAi, has been successfully silenced using this method *(17)*. In addition to its use in *C. elegans*, microparticle bombardment has been used to transform other nematode species, including *Ascaris* and *Litomosoides sigmodontis (18,19)*.

The major advantages of the microparticle bombardment approach over the germ-line injection method are its technical simplicity and high success rate. The equipment is simple to set up and use, and the population-based approach allows for the scaling necessary to obtain rare low-copy and homologous integrants *(15,16)*. Because it has become simpler to create integrated lines using microparticle bombardment, researchers have been better able to study gene expression in both the germ line and soma, including studies of genes that are dose sensitive *(12,20–24)*.

Microparticle bombardment transformation of *C. elegans* does have limitations that should to be considered before using this technology. First, transformation of a population rather than individual hermaphrodites requires a good selectable marker. Owing to genetic interactions between the selectable marker and gene of interest, it may be difficult to create healthy double mutants that can be transformed. Incomplete rescue of the mutant phenotype associated with the selectable marker or overexpression of the selectable marker gene can also alter the phenotype of the strain being characterized, making gene expression and localization studies difficult to interpret. During the creation of low-copy integrated lines, expression of the gene of interest may not reflect that of the endogenous gene, owing to higher copy number and position effects associated with the integration event. The integrated exogenous DNA may also interfere with the expression of other genes located at the site of integration. Finally, despite some success, researchers still encounter problems expressing genes in the germ line, including altered expression patterns and silencing (Strome, S., Seydoux, G., and Desai, A., personal communication, 2005). To overcome some of these concerns, researchers are encouraged to backcross and analyze multiple transgenic lines.

Two general types of cotransformation markers have been used successfully in microparticle bombardment experiments: selectable markers that rescue or produce a mutant phenotype and those that express green fluorescent protein (GFP) in a specific tissue. Examples of the former include those that rescue *unc-119, pha-1*, *dpy-20*, and *spe-26* mutant phenotypes or express dominant *rol-6*. Examples of the latter include the plasmids pGK10 and pPD118.33, which carry GFP promoter fusions (**Table 1**). Each of these cotransformation markers has distinct advantages and disadvantages that might influence a researcher's choice, including the difficulty in growing the strain, the simplicity of the selection strategy, and the possibility of gene interactions. Note that because integrated lines are rarer but more stable than extrachromosomal array lines, a selection strategy that identifies transformed individuals from F_2 or later generations is more likely to yield these types of transformants than one that relies on selection during the F_1 generation. Most of the cotransformation markers listed in **Table 1** have been used with both plasmids and cosmids to

Table 1
Cotransformation Markers Used in Microparticle Bombardment Experiments

Transformation plasmid	Phenotype	Comments[a]	References
pDPMM0016b	Rescues *unc-119(ed3)*	*unc-119* can be difficult to grow. Select for live, mobile hermaphrodites 10–14 d after transformation. *unc-119(ed3)* cannot form dauers.	*15,25*
pBx	Rescues *pha-1(e2123ts)*	Temperature sensitive strain is easy to grow. Select for live hermaphrodites at the restrictive temperature. *pha-1(e2123ts)* can revert, so check strain integrity prior to transformation.	Schnabel R. and Struck, C., personal communication; and **ref. 26**
pJV145	Rescues *spe-26(hc138ts)*	Temperature sensitive strain is easy to grow. Select for fertile hermaphrodites growing at the restrictive temperature.	Smith, H. E. and Ward, S., personal communication; and **refs 27–29**
pMH86	Rescues *dpy-20 (e1282ts)*	Select for non-Dpy in a population of Dpy hermaphrodites.	
pRF4	Expresses *rol-6*; Dominant ROL phenotype	Select for rollers in a population of wild-type.	Schnabel R. and Struck, C., personal communication; and **ref. 30**
pGK10	Expresses GFP in muscles using the Serca gene promotor	Select for GFP expression. Limited use when examining novel GFP expression patterns.	Schnabel R. and Struck, C., personal communication; and **ref. 31**
pPD118.33	Expresses GFP in the pharynx using *myo-2* promotor.	Select for GFP expression. Limited use when examining novel GFP expression patterns.	Schnabel R. and Struck, C., personal communication

[a]Because microparticle bombardment techniques use large number of worms, the food supply can be rapidly depleted after transformation, causing dauer arrest prior to the selection. To avoid this problem, use more plates, move worms, or regularly add *Escherichia coli*.

produce lines carrying extrachromosomal arrays and low-copy integrants. Contrary to early reports, the cotransformation marker and gene of interest need not be on a single plasmid to produce integrated lines (**refs. *15* and *20*;** and Schwartz, H., Miskevich, F., Constantine-Paton M., and Horvitz, H. R., personal communication, 2005).

Four distinct methods for microparticle bombardment transformation of *C. elegans* have been described in the literature, utilizing the Bio-Rad Biolistic® PDS-1000/He Particle Delivery system, with or without the Hepta adapter, the Bio-Rad Helios Gene Gun system, or an alternative microparticle bombardment apparatus (**refs. *13–16*** and Schwartz, H., et al., personal communication, 2005; Schnabel, R. and Struck, C., personal communication; and Shaham, S., personal communication, 2005).

As the cost of equipment, ease of use, the scale of the transformations and other factors vary, researchers will need to determine which protocol is most suitable for their laboratory. Based on current data, the transformation efficiencies for each device are similar. However, comparisons are made difficult by the fact that a number of variables can influence transformation efficiency. Extrachromosomal lines are easier to produce than integrated lines. Genes expressed in the germ line tend to have lower transformation frequencies than those expressed in the soma. It may also be more difficult express genes that are dose sensitive, because even in integrated lines gene expression levels may not be identical to the wild-type. For example, using the Bio-Rad Biolistic PDS-1000/He Particle Delivery system and rescue of *unc-119(ed3)* as the selectable marker, integrated lines were obtained at a frequency range of 9–35%, depending on the second gene on the construct *(15)*. There is also some variability in the frequency with which a gene is expressed with a given cotransformation marker. When using the alternative microparticle bombardment apparatus and pRF4 as a cotransformation marker, for example, almost 100% of the identified strains expressed the second gene. However, using rescue of *pha-1* as the selection, 70% of the lines expressed the second gene (Schnabel, R. and Struck, C., personal communication). Because the precise conditions of the microparticle bombardment apparatus may also influence transformation efficiency, researchers are encouraged to optimize the protocol in their laboratory by performing control experiments with a well-characterized cotransformation marker. In this chapter, we include descriptions of each of the four microparticle bombardment protocols to introduce exogenous DNA into *C. elegans*.

2. Materials
2.1. C. elegans *Strains and Growth Conditions*

1. The precise conditions for maintaining and growing *C. elegans* strains will depend on the selection strategy you choose (**Table 1**). Most strains can be maintained at

20°C on 60 mm nematode growth medium (NGM) plates seeded with OP50 bacteria *(32)*. However, strains carrying temperature-sensitive mutations will need to be grown at the appropriate temperature. For growing large quantities of worms for bombardment, use liquid cultures, 100-mm NGM, opti-gro, enriched peptone, or egg plates completely spread with OP50, HB101, or NA22 bacteria. Addition of nystatin to plate media will reduce growth of fungi *(33)*. Just prior to bombardment, young adults are transferred to relatively dry NGM or enriched plates (40-, 60-, or 100-mm, depending on the specific protocol), seeded with *Escherichia coli*. Transfer worms between plates using M9 buffer.

2. Enriched peptone plates: 1.2 g sodium chloride, 20 g peptone, 25 g agar in 1 L H_2O. Autoclave. Cool to 55°C then add sterile 1 mL cholesterol (5 mg/mL in EtOH), 1 mL 1 *M* $MgSO_4$, and 25 mL 1 *M* potassium phosphate (pH 6.0).

2.2. Microparticle Bombardment Transformation

1. Bio-Rad Biolistic® PDS-1000/He particle delivery system. One method to transform *C. elegans* utilizes the Bio-Rad Biolistic PDS-1000/He particle delivery system (Bio-Rad). This basic transformation protocol can be scaled up by using the Hepta adapter. To familiarize yourself with the equipment and its requirements, read and follow the manufacturer's directions *(15,16,34,35)*. Supplies required to operate the equipment include: 1350 psi rupture discs for the conventional system or 1500–2000 psi rupture discs if using the Hepta adapter, macrocarriers, and stopping screens. Macrocarriers, macrocarrier holders, Hepta adapter, and screens should be sterilized in EtOH or autoclaved prior to each use. Wipe down the bombardment chamber with 70% ethanol. Reagents and solutions required for preparing gold beads include: 1-μ gold bead microcarriers, 0.1 *M* spermidine (free base, tissue culture grade; filter-sterilized; store frozen at –20°C), sterile 2.5 *M* $CaCl_2$, dehydrated ethanol, and 50% sterile glycerol.

2. An alternative microparticle bombardment device. The Schnabel research group has developed a microparticle DNA transformation protocol for *C. elegans* using an apparatus originally designed for transformation of plants (**refs.** *13,14,36,* and *37*; and Schnabel, R. and Struck, C., personal communication). This device, which can be constructed by a researcher with access to good electronics and mechanical workshops, is an attractive alternative to the Bio-Rad devices because of its lower cost. Instructions for obtaining parts, setting up the device, and operating it, along with a list of required accessories, are available from the Schnabel lab home page (www.tu-bs.de/institute/genetik/schnabel/ce-home). Researchers are encouraged to contact Ralf Schnabel for additional information. Reagents and solutions required for preparing gold beads include: 0.3–3.0-μ gold beads (Chempur, Karlsuhe, Germany), 50 m*M* spermidine, sterile 1 *M* $CaCl_2$, 20 mg/mL polyvinylpyrrolidone (PVP) in H_2O. Store at 4°C. Make a working stock of 0.1 mg/mL PVP in ethanol just prior to use.

3. Bio-Rad Helios Gene Gun. The BioRad Helios Gene Gun can also be used to transform *C. elegans*. To familiarize yourself with the equipment and its require-

ments, read and follow the manufacturer's instructions (Schwartz, H., et al., personal communication; and **ref. *38***). Supplies required to operate the equipment include: Gold-Coat tubing, cartridge collection/storage vials, and desiccant pellets. Reagents and solutions required for preparing the gold beads include: 1-μm diameter gold beads, 50 μM spermidine, sterile 1 M CaCl$_2$, 20 mg/mL PVP in ethanol. Store at 4°C. Make a working stock of 0.1 mg/mL PVP in ethanol just prior to use, dehydrated ethanol.

2.3. Preparation of DNA for Transformation

The DNA required for transformation can be prepared using a standard alkaline lysis protocol *(39)* or a DNA purification kit such as Qiagen (Valencia, CA). DNA should be concentrated (~1 mg/mL) and of high purity. Potential cotransformation markers are listed in **Table 1**. Note that the cotransformation marker and gene of interest need not be on the same plasmid (**ref. *20***; and Schwartz, H., et al., personal communication).

3. Methods
3.1. Growing C. elegans Strains for Transformation

The following protocols are for preparing large quantities of *C. elegans* prior to microparticle bombardment transformation. Specific conditions will vary depending on the selection strategy and strain you are transforming (**Table 1**). Because each of the microparticle bombardment transformation protocols described in this chapter requires a specific number of worms for a transformation, instructions for each protocol are included.

1. Create a master plate of the worm strain you have chosen by evenly dispersing worms on an enriched media plate completely spread with bacteria. Let the worms grow until they have starved (~7 d for *unc-119[ed3]*). It is particularly useful to maintain master plates for *unc-119* mutant strains, which are slow growing.
2. For the Bio-Rad Biolistic PDS-1000, Helios, or alternative protocols, transfer approximately one-sixth of the starved L1 larva from the master plate onto a 100-mm enriched media plate and grow at appropriate temperature until they become young adults (*see* **Note 1**). Each plate will be used for approx 1–2 bombardment(s), so it is useful to prepare five or more plates for each transformation.
3. For the Hepta adapter protocol, use M9 to transfer approximately one-sixth of the starved L1 larva from the master plate onto each of six 100-mm enriched peptone plates and grow to starved L1s. Wash off worms in 60 mL M9 and transfer to 60 enriched peptone plates. Grow until they become young adults (*see* **Note 1**).
4. Wash off the young adults with M9 buffer and transfer to centrifuge tube. Pellet adults by leaving tube on bench for 5 min or spinning at low speed for 1 min. Remove supernatant and use a Pasteur pipet or a 200-μL micropipet tip with the end cut off to transfer worm pellet to a *dry* bombardment plate, as indicated for the specific protocol you are using.

5. If using the Bio-Rad Biolistic PDS-1000 or Helios system, transfer worm pellet onto dry 60-mm NGM plates seeded with a 20-mm patch of *E. coli*. A 0.25-mL worm pellet, harvested from five 100-mm rich media plates, is sufficient for five to six bombardments.

6. If using the Bio-Rad Hepta adapter, transfer worm pellet onto dry 100-mm rich media plates completely seeded with *E. coli*. A 2- to 4-mL worm pellet, harvested from sixty 100-mm plates, is sufficient for six to seven bombardments.

7. If using the alternative device, transfer worm pellet onto dry ice-cold 40-mm NGM plates thinly seeded with OP50. A 0.1-mL worm pellet, harvested from two to three 100-mm plates, is sufficient for five bombardments. The thin lawn of bacteria on the plate should be covered with approximately two layers of worms.

8. Leave lid off of the bombardment plates to allow them to dry. To prevent mobile strains from moving off the bacterial lawn, prechill plates to 4°C and keep on ice.

3.2. Microparticle Bombardment Transformation Protocols Using the Biolistic PDS-100/He Particle Delivery System With or Without the Hepta Adaptor

See **refs. 15,16,34,** and **35**; and Shaham, S., personal communication.

3.2.1. Preparation of Beads for Transformation

1. Add 1 mL 70% ethanol to 18 mg of 1-µ gold beads in a siliconized microfuge tube. Vortex for 5 min, allow particles to settle for 15 min, spin 3–5 s in microfuge and remove supernatant (*see* **Note 2**).

2. Add 1 mL deionized water. Vortex 1 min, allow particles to settle 1 min, spin 3–5 s in microfuge, and remove supernatant.

3. Repeat **step 2** three times.

4. Add 300 µL 50% glycerol to gold bead pellet and vortex until well suspended (*see* **Note 3**).

5. Using a siliconized microfuge tube, add the following reagents per bombardment (*see* **Note 4**):
   a. 10 µL Prepared gold beads in 50% glycerol.
   b. 1 µL DNA (concentration ~1 mg/mL; *see* **Note 5**).
   c. 10 µL of 2.5 *M* CaCl$_2$.
   d. 4 µL of 0.1 *M* spermidine.

   Vortex for 1 min between each addition and at least 3 min after the final addition. Allow beads to settle for 1 min, spin 3–5 s in microfuge and remove supernatant.

6. Resuspend pellet in 30 µL 70% EtOH/bombardment, spin briefly, and remove supernatant.

7. Resuspend pellet in 30 µL 100% EtOH/bombardment, spin briefly, and remove supernatant.

8. Resuspend in 10 µL 100% EtOH/bombardment. Vortex at least 3 min (*see* **Note 6**).

9. If not using the Hepta adapter, spread approx 10 μL gold bead/DNA suspension over central region of a macrocarrier and let dry. Prepare one macrocarrier per bombardment.

10. If using the Hepta adapter, scale up preparation by preparing three tubes of DNA/beads each of which contains a final volume of 100 μL DNA/bead suspension (e.g., for each tube increase by 10-fold the reagents listed in **steps 5–8**). To each of the seven macrocarriers used per bombardment, add approx 6–7 μL of this final DNA suspension to the central region and let dry. Repeat for each of six bombardments.

3.2.2. Transformation Without the Hepta Adapter

1. Set up the Bio-Rad Biolistic PDS-1000/He particle delivery system without the Hepta adapter, following the manufacturer's instructions. Be sure you are familiar with the equipment and supplies, test the equipment properly, and follow all safety guidelines *(34)*.

2. Set up a 0.25-in. gap distance between the rupture disc holder and the macrocarrier launch assembly.

3. Place a 1350 psi rupture disc into the retaining cap and tighten with the provided torque wrench.

4. Using the macrocarrier insertion tool, place DNA-coated macrocarrier into macrocarrier holder and screw into the macrocarrier launch assembly according to the manufacturer's instructions *(34)*.

5. Remove lid and place worm plate on the second target shelf from the bottom.

6. Evacuate chamber to 27 in. Hg. Press and hold fire button until rupture disc breaks. Release vacuum and remove worm plate (*see* **Note 7**).

3.2.3. Transformation With the Hepta Adapter

1. Set up the Bio-Rad Biolistic PDS-1000/He particle delivery system with the Hepta adapter following the manufacturer's instructions *(34,35)*.

2. Place the seven macrocarriers onto the macrocarrier holder with seating tool, add DNA suspension, and let dry.

3. Put stopping screen and macrocarrier holder together.

4. Soak a 1500- to 2000-psi rupture disc in isopropanol for 3–5 s, place in the retaining cap of the Hepta adapter, and tighten the adapter onto the chamber.

5. Place macrocarrier in chamber according to manufacturer's instructions *(35)*.

6. Secure worm plate in lowest shelf of the chamber with tape (*see* **Note 8**).

7. Evacuate chamber to 27 in. Hg, press fire button, and hold until disc ruptures. Release vacuum and remove plate.

3.2.4. Postbombardment Care of Worms

1. After bombardment, allow worms to recover for approx 1 h.

2. If using the Helios Gene Gun or the Bio-Rad Biolistic PDS-1000/He particle delivery system without the Hepta adapter, wash worms off of bombardment plate with M9 buffer and transfer to two or more 100-mm enriched plates per

bombardment. Save original bombardment plates, as these will also yield trans-formed lines.

3. If using the Hepta adapter, wash off worms from six to seven bombardment plates with M9 buffer, pellet, and transfer to 80 100-mm enriched media plates.

4. Incubate plates at appropriate temperature. Timing and identification of trans-formed lines will depend on the selection strategy you use. GFP expression of germ-line genes may be enhanced by growth at 25°C (Strome, S. and Seydoux, G., personal communication; *see* **Note 9**).

3.3 Transformation of C. elegans
Using the Alternative Microparticle Bombardment Device

See **ref. *14*** and Schnabel R. and Struck, C., personal communication.

3.3.1. Preparation of Gold Beads for Transformation

This protocol prepares enough beads for 10 bombardments.

1. Add 100 μL 50 m*M* spermidine to 1 mg gold (0.3- to 3-μm diameter) in a 1.5-mL microfuge tube. Vortex, then sonicate for 5 s.

2. Add 16 to 32 μg DNA and incubate for 10 min, mixing occasionally (*see* **Note 5**).

3. Add deionized water to a final volume of 360 μL. Vortex and then incubate for 10 min, mixing occasionally.

4. Add 100 μL 1 *M* CaCl$_2$, in drops to reduce clumping gold beads. Vortex and incubate for 10 min at room temperature to precipitate the DNA.

5. Microfuge 15 s (15,000*g*) and remove supernatant.

6. Wash pellet three times with 1 mL ethanol.

7. Resuspend DNA/gold pellet in 200 μL of 0.1 mg/mL PVP solution (*see* **Note 6**).

8. Use 20 μL of the resuspended gold bead solution per bombardment.

3.3.2. Transformation

1. Calibrate the bombardment device by shooting at a piece of filter paper and draw-ing a crosshair through the target area (*see* **Note 11**).

2. Set the He pressure valve on the gun to 8 bar (10 on the valve on the bottle).

3. Load the steel grid with the DNA-coated gold suspension in the gun.

4. Center the worm plate on the crosshairs 120 mm from the filter holder and remove plate lid just before closing the chamber (*see* **Note 12**).

5. Start to evacuate the chamber. When the vacuum is between 0.4 and 0.5 bar, trigger the gun. The pulse time should be between 10 and 30 ms (*see* **Note 7**).

6. Release the vacuum, immediately cover the worm plate, and place on ice.

3.3.3. Postbombardment Care of Worms

1. Cut the agar of each bombardment plate into six sectors and put each piece on a fresh 100-mm enriched media plate. Once the plates have warmed, check them under a dissecting scope. For a successful bombardment, approx 50% of the worms should be dead after one shot.

2. Incubate plates at appropriate temperature. Timing and identification of transformed lines will depend on the selection strategy you use (*see* **Note 9**).

3.4. Transformation Using the Bio-Rad Helios Gene Gun

Schwartz, H., et al., personal communication; and *see* **ref. *38***.

3.4.1. Preparation of Beads for Transformation

1. Make up a 1-mg/μL stock of 1-μ gold beads in 50 μ*M* spermidine.
2. Resuspend gold by vortexing. Pipet 15 μL into a microfuge tube. Add 85 μL 50 μ*M* spermidine. Vortex mixture and sonicate briefly to break up clumps.
3. Add 50 μg DNA (*see* **Note 5**) to beads and mix by vortexing for 5–10 s.
4. Add 100 μL 1 *M* CaCl$_2$ to sample in small drops, mixing continuously to prevent DNA-coated beads from clumping. Allow DNA to precipitate at room temperature for 10 min. Spin in a microcentrifuge for approx 15 s to pellet gold. Remove and discard supernatant. Resuspend pellet in the remaining supernatant by vortexing briefly.
5. Wash with 1 mL ethanol, vortexing well, and centrifuging approx 5 s each time. Sonicate briefly, if necessary. Repeat 5–10 times, until gold is well suspended. Remove the supernatant from the final wash (*see* **Note 6**).
6. Resuspend the DNA/bead pellet in 200 μL of freshly-made 0.1-mg/mL PVP solution. Transfer to a 15-mL polypropylene screw-top centrifuge tube. Because some of the DNA/bead pellet will remain in the tube, repeat with another 200-mL PVP solution and transfer to the same 15-mL tube. Repeat as necessary. Bring final volume to 3 mL with PVP solution (*see* **Note 13**).
7. Load the DNA/gold bead solution into the Gold-Coat tubing using the tubing Prep Station, according to the manufacturer's instructions *(38)*.
8. Place a scintillation vial containing a desiccant pellet in the tubing cutter. Cut tubing into 0.5-in. segments. Cap the vial tightly, label it, and seal with Parafilm. The cartridges are stable for up to 9 mo stored at 4°C.

3.4.2. Transformation

1. Activate the Helios Gene Gun using an empty cartridge holder according to the manufacturer's instructions *(38)*. Alternatively, reserve one empty slot in a filled cartridge holder and use this to pressurize the system at 200 psi (*see* **Note 14**).
2. Load cartridges into cartridge holder and load into the Helios Gene Gun.
3. Put a plate of worms on a flat surface and remove the cover. Hold the gun flush to the surface of the agar, centered over the worms (*see* **Note 15**). Fire the gun.
4. Repeat **step 3** until you have fired all your cartridges.
5. Remove cartridge holder from the Helios Gene Gun, depressurize, and shut down system.

3.4.3. Postbombardment Care of Worms

Proceed as described in the Bio-Rad Biolistic protocol (*see* **Subheading 3.2.4.**).

4. Notes

1. Young adults with 5–10 eggs inside transform most efficiently.
2. Preparing the gold beads for bombardment involves a lot of vortexing. We strongly suggest a turbomixer attachment for your vortexer (Fisher Scientific). A tiny benchtop microfuge is good for pelleting gold beads, as they are less likely to compact.
3. Protocol can be stopped at this point. Prepared beads are good for at least 2 wk stored at 4°C or room temperature. The quantity prepared here is sufficient for 25 bombardments. Vortex prepared gold beads for at least 5 min prior to use to ensure they are well suspended.
4. The amounts described in this section are for a single bombardment, although it is more practical to perform five or more bombardments.
5. To optimize transformation frequencies for each construct, try a range of DNA amounts (0.5 to 2X). When transforming with two constructs, use equal amounts of DNA in a similar concentration range.
6. It is essential the beads do not clump. If beads clump, use additional vortexing, pipeting, or sonication until all large clumps are broken up. Test a resuspension by viewing 1–2 µL on a slide using a compound microscope. You should see single beads and small clusters.
7. It is recommended that you test these parameters on your microparticle bombardment device by first rescuing a known gene (e.g., perform several transformations using the *unc-119* or *pha-1* selection systems). If you do not observe a reasonable transformation frequency, adjust the parameters.
8. Alternatively, one can use 1350/1550 rupture discs; secure worm plate to second shelf from bottom.
9. One will observe transformed individuals in the F_1 generation, most of which will carry extrachromosomal arrays. The F_2 and later generations will have higher frequencies of stable lines. To select for homologous integrants, clone out multiple individuals from a positive plate. Look for clones that segregate 75 or 100% progeny with the desired phenotype.
10. Use genetic crosses to confirm integration events.
11. Calibration should be repeated from time to time.
12. The cover of the chamber should not be fixed! For your safety, it will lift if the pressure becomes too high.
13. The gold particles can be used immediately or stored for up to 2 mo at –20°C, tightly sealed. Allow tube to come to room temperature before breaking seal.
14. Bio-Rad recommends 120–180 psi at a 2-cm distance for the parasitic nematode *Litomosoides sigmodontis* and 300 psi used at a 3-cm distance for *C. elegans* (*19*). Because 250 psi was found to be less efficient than 200 psi in some experiments (Schwartz, H., et al., personal communication) researchers may want to try several parameters to see what works best.
15. Do not worry about breaking the agar. To avoid contamination, clean the barrel liner with ethanol and air-dry.

Acknowledgments

References

1. Mello, C. and Fire, A. (1995) DNA transformation. *Methods Cell Biol.* **48,** 451–482.
2. Stinchcomb, D. T., Shaw, J. E., Carr, S. H., and Hirsh, D. (1985) Extrachromosomal DNA transformation of *Caenorhabditis elegans*. *Mol. Cell. Biol.* **5,** 3484–3496.
3. Fire, A. and Waterston, R. H. (1989) Proper expression of myosin genes in transgenic nematodes. *EMBO J.* **8,** 3419–3428.
4. Mello, C. C., Kramer, J. M., Stinchcomb, D., and Ambros. V. (1991) Efficient gene transfer in *C. elegans*: extrachromosomal maintenance and integration of transforming sequences. *EMBO J.* **10,** 3959–3970.
5. MacMorris, M., Spieth, J., Madej, C., Lea, K., and Blumenthal, T. (1994) Analysis of the VPE Sequences in the *Caenorhabditis elegans vit-2* promotor with extrachromosomal tandem array-containing transgenic strains. *Mol. Cell. Biol.* **14,** 484–491.
6. Hsieh, J., Liu, J., Kostas, S. A., Chang, C., Sternberg, P. W., and Fire, A. (1999) The Ring finger/B-Box factor TAM-1 and a retinoblastoma-like protein LIN-35 modulate context-dependent gene silencing in *Caenorhabditis elegans*. *Genes Dev.* **13,** 2958–2970.
7. Kelly, W. G., Xu, S., Montgomery, M. K., and Fire, A. (1997) Distinct requirements for somatic and germline expression of a generally expressed *Caenorhabditis elegans* gene. *Genetics* **146,** 227–238.
8. Krause, M., Harrison, S. W., Xu, S. Q., Chen, L., and Fire, A. (1994) Elements regulating cell and stage-specific expression of the *C. elegans* MyoD family homolog *hlh-1*. *Dev. Biol.* **166,** 133–148.
9. Fire, A. (1986) Integrative transformation of *Caenorhabditis elegans*. *EMBO J.* **5,** 2673–2680.
10. Spieth, J., MacMorris, M., Broverman, S., Greenspoon, S., and Blumenthal, T. (1988) Regulated expression of a vitellogenin fusion gene in transgenic nematodes. *Dev. Biol.* **130,** 285–293.
11. Barrett, P. L., Fleming, J. T., and Gobel, V. (2004) Targeted gene alteration in *Caenorhabditis elegans* by gene conversion. *Nat. Genet.* **36,** 1231–1237.
12. Strome, S., Powers, J., Dunn, M., et al. (2001) Spindle dynamics and the role of gamma-tubulin in early *Caenorhabditis elegans* embryos. *Mol. Biol. Cell* **12,** 1751–1764.
13. Jackstadt, P., Wilm, T. P., Zahner, H., and Hobom, G. (1999) Transformation of nematodes via ballistic DNA transfer. *Mol. Biochem. Parasitol.* **103,** 261–266.

14. Wilm, T., Demel, P., Koop, H. U., Schnabel, H., and Schnabel, R. (1999) Ballistic transformation of *Caenorhabditis elegans*. *Gene* **229,** 31–35.

15. Praitis, V., Casey, E., Collar, D., and Austin, J. (2001) Creation of low-copy integrated transgenic lines in *Caenorhabditis elegans*. *Genetics* **157,** 1217–1226.

16. Berezikov, E., Bargmann, C., and Plasterk, R. (2004) Homologous gene targeting in *Caenorhabditis elegans* by biolistic transformation. *Nucleic Acids Res.* **32,**

17. Johnson, N. M., Behm, C. A., and Trowell, S. C. (2005) Heritable and inducible gene knockdown in *C. elegans* using Wormgate and the ORFeome. *Gene* **10,** 26–34.

18. Davis, R. E., Parra, A., LoVerde, P. T., Ribeiro, E., Glorioso, G., and Hodgson, S. (1999) Transient expression of DNA and RNA in parasitic helminths by using particle bombardment. *Proc. Natl. Acad. Sci. USA* **96,** 8687–8692.

19. Jackstadt, P., Zahner, H., and Hobom, G. (1999) Bolistic particle delivery. Transformation nematodes with the Helios™ gene gun. Bio-Rad Technical Note 2433.

20. Askjaer, P., Galy, V., Hannak, E., and Mattaj. I. (2002) Ran GTPase cycle and importins alpha and beta are essential for spindle formation and nuclear envelope assembly in living *Caenorhabditis elegans* embryos. *Mol. Biol. Cell* **13,** 4355–4570.

21. Trappe, R., Schulze, E., Rzymski, T., Frode, S., and Engel, W. (2002) The *Caenorhabditis elegans* ortholog of human PHF5a shows a muscle-specific expression domain and is essential for *C. elegans* morphogenetic development. *Biochem. Biophys. Res. Commun* . **297,** 1049–1057.

22. Kirkham, M., Muller-Reichert, T., Oegema, K., Grill, S., and Hyman, A. (2003) SAS-4 is a *C. elegans* centriolar protein that controls centrosome size. *Cell* **112,** 575–587.

23. Pellettieri, J., Reinke, V., Kim, S., and Seydoux, G. (2003) Coordinate activation of maternal protein degradation during the egg-to-embryo transition in *C. elegans*. *Dev. Cell* **5,** 451–462.

24. Grad, L., and Lemire, B. (2004) Mitochondrial complex I mutations in *Caenorhabditis elegans* produce cytochrome c oxidase deficiency, oxidative stress and vitamin-responsive lactic acidosis. *Hum. Mol. Gen.* **13,** 303–314.

25. Maduro, M. and Pilgrim, D. (1995) Identification and Cloning of *unc-119*, a gene expressed in the *Caenorhabditis elegans* nervous system. *Genetics* **141,** 977–988.

26. Granato, M., Schnabel H., and Schnabel, R. (1994) *pha-1*, a selectable marker for gene-transfer in *C. elegans*. *Nucleic Acids Res.* **22,** 1762–1763.

27. Varkey, J., Muhlrad, P., Minniti, A., Do B.K., and Ward, S. (1995) The *Caenorhabditis elegans spe-26* gene is necessary to form spermatids and encodes a protein similar to the actin-associated proteins Kelch and Scruin. *Genes Dev.* **9,** 1074–1086.

28. Clark, D. V., Suleman, D. S., Beckenbach, K. A., Gilchrist E. J., and Baillie, D. L. (1995) Molecular cloning and characterization of the *dpy-20* gene of *Caenorhabditis elegans*. *Mol. Gen. Genet.* **247,** 367–378.

29. Kniss, S. (2002). The role of the sue genes in the neuronal regulation of *C. elegans* behavior. PhD Thesis, University of Chicago, Chicago, IL.

30. Kramer, J. M., French, R. P., Park, E.C., and Johnson, J. J. (1990) The *Caenorhabditis elegans rol-6* gene, which interacts with the *sqt-1* collage gene to determine organismal morphology, encodes a collagen. *Mol. Cell Biol.* **10,** 2081–2089.

31. Zwaal, R. R., Van Baelen, K., Groenen, J. T., et al. (2001) The sarco-endoplasmic reticulum Ca2+ ATPase is required for development and muscle function in *Caenorhabditis elegans*. *J. Biol. Chem.* **276,** 43,557–43,563.

32. Brenner, S. (1974) The genetics of *Caenorhabditis elegans*. *Genetics* **77,** 71–94.

33. Lewis, J. A. and Flemming, J. T. (1995) Basic culture methods, in Caenorhabditis elegans*: Modern Biological Analysis of an Organism,* (H. F. Epstein and D. C. Shakes, eds.), Academic Press, San Diego, CA, pp. 3–29.

34. Bio-Rad (1997) Bio-Rad Biolistic® PDS-1000/He Particle Delivery System Instruction Manual, Hercules, CA.

35. Bio-Rad. Bio-Rad Hepta Adapter Instruction Manual, Hercules, CA.

36. Finer, J. J., Vain, P., Johns M. W., and McMullen, M. D. (1992) Development of the particle gun for the delivery to plant cells. *Plant Cell Rep.* **11,** 323–328.

37. Takeuchi, Y., Dotson, M., and Keen, N. T. (1992) Plant transformation: a simple particle bombardment device based on flowing helium. *Plant Mol. Biol.* **18,** 835–839.

38. Bio-Rad (2001) Bio-Rad Helios Gene Gun System Instruction Manual, Hercules, CA.

39. Sambrook, J., Fritsch, E. F., and Maniatis. T. (1989) *Molecular Cloning: A Laboratory Manual, 2nd Ed.*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

8

Construction of Plasmids for RNA Interference and In Vitro Transcription of Double-Stranded RNA

Lisa Timmons

Summary

Double-stranded RNA (dsRNA)-induced gene silencing in *Caenorhabditis elegans* involves the manufacture and delivery of defined sequences of dsRNA to the organism, followed by a careful monitoring for loss-of-function phenocopies in treated animals. In this chapter, we describe how to generate DNAs that can be used as templates for transcription of dsRNA.

Key Words: Double-stranded RNA; dsRNA; single-stranded RNA, ssRNA; RNAi; RNA silencing; feeding; soaking; injection; HT115; HT115(DE3); transgene; hairpin RNA; RNaseIII; transcription; functional genomics.

1. Introduction

The directed isolation of mutant strains of *Caenorhabditis elegans* using genetic screens*,* coupled with phenotypic analysis and identification of the mutated gene, has proven a valuable means to elucidate gene function, to link a gene to a cellular or developmental process, and to unravel genetic pathways. Such forward genetic approaches are straightforward, yet can be time-consuming and may fail to identify some genes that function in the process of interest. In 1998, Fire and Mello discovered that a specific sequence of double-stranded RNA (dsRNA) could lead to silencing of the corresponding gene when injected into worms, a phenomenon they termed RNA interference (RNAi *[1]*). Reverse genetics approaches using RNAi can easily uncover functions for a particular gene sequence, and with the availability of the completed sequence of the *C. elegans* genome, virtually every gene can be targeted using RNAi.

RNAi in *C. elegans* entails the manufacture of dsRNA, delivery of dsRNA to worms, and extensive phenotypic analysis of affected animals. dsRNAs can
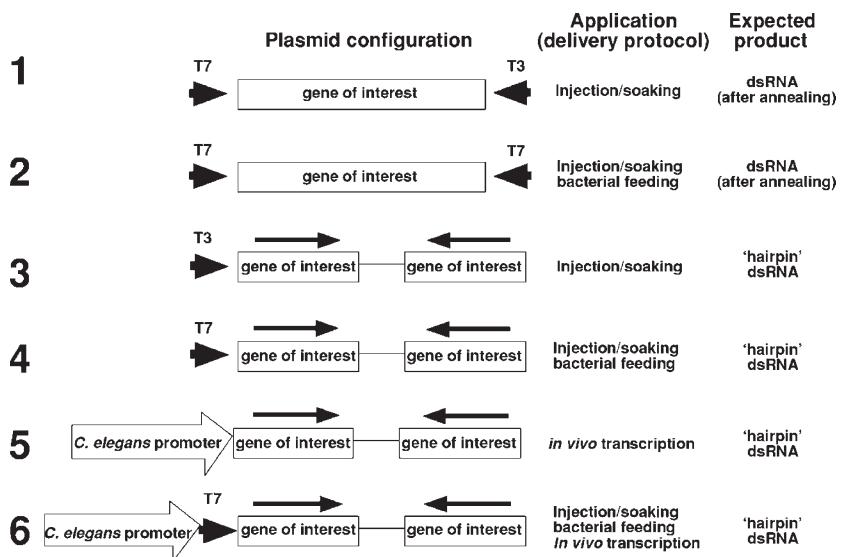
Fig. 1. Plasmid configurations for double-stranded RNA (dsRNA) synthesis. The relative positioning and orientations of gene sections and promoters are indicated. As routine experiments designed to uncover the function of a particular gene are performed, labs often generate a number of different constructs that can be used in RNA interference experiments. Each of the configurations depicted here can be used in at least one dsRNA delivery method. Injection and soaking require in vitro transcription; bacterial feeding requires transformation of the indicated plasmid into HT115(DE3) cells; in vivo delivery requires injection of the plasmid, with transformation marker, into *Caenorhabditis elegans* to generate transgenic lines. *Construct 1* can be used in in vitro transcription reactions—T7 and T3 polymerase are required to produce both strands. *Construct 2* is used in the feeding protocol and is also a convenient configuration for producing dsRNA from polymerase chain reaction-generated templates—T7 primer sites can be incorporated into the amplification primers (**Fig. 2**). *Construct 3* is useful for in vitro transcription of hairpin dsRNAs using a T3 polymerase, whereeas *Construct 4* uses T7. *Constructs 5* and *6* allow trancription of a hairpin dsRNA in *C. elegans* cells when transformed into the animal. Short, bold arrows represent bacteriophage promoters. Longer, bold arrows indicate the orientation of the gene fragment used in dsRNA hairpin production.

be introduced into the organism using a number of different protocols (*see* Chapter 9). *Injection (1)* and *soaking (2)* delivery methods require production of dsRNAs by in vitro transcription. In vitro transcription utilizes a DNA template with bacteriophage promoter sequences that are properly oriented with respect to the gene of interest (**Fig. 1**). Polymerase chain reaction (PCR) prod-

ucts, as well as plasmids, can be used as templates for in vitro transcription reactions. A plasmid is required for dsRNA delivery using bacteria in *feeding* protocols *(3)*; dsRNA is made in bacteria that are subsequently ingested by worms. Feeding plasmids are configured with two T7 promoter sites in inverted configuration flanking the gene-of-interest (**Fig. 1**, *Construct 2*). Alternatively, a single T7 promoter site preceding an inverted repeat of the gene-of-interest can be used in the feeding protocol (**Fig. 1**, *Construct 4*). (T3 promoters [**Fig. 1**, *Constructs 1,3*] do not function in the bacterial feeding system.) Finally, in vivo delivery is accomplished as dsRNA is transcribed in the cells of the worm from transformed DNAs.

When selecting a gene segment to use as template for dsRNA production, it is not necessary, nor is it always advisable, to use the entire coding region. One should consider the following:

1. Region: dsRNA trigger sequences should be derived from coding regions and not introns. Because many *C. elegans* genes are interspersed with small introns, it may be necessary to obtain a cDNA clone in order to obtain a contiguous stretch of exonic sequence sufficient to trigger robust RNAi.
2. Length: longer dsRNAs (in the 500- to 1000-bp range) may be more effective in eliciting RNAi because they are fragmented into a large number of effector short interfering RNA (siRNA) molecules. However, when longer sense and antisense strands are synthesized in separate tubes, efficient annealing of the two strands may prove difficult. Two to three hundred base pairs of sequence is often sufficient for RNAi and in most cases, strands of this length anneal fairly readily.
3. Specificity: it is possible to target multiple RNAs for destruction using only one dsRNA trigger, and there are no reliable methods that can predict cross-interference. BLAST or similar programs can be used to find related sequences—70–80% identity over 200 bp of sequence could result in such off-target silencing.

The importance and value of RNAi technology in *C. elegans* genetics is revealed by the commercial availability of resources (*see* **Subheading 2.**), imparting an increased simplicity to RNAi experiments. Finally, please remember that although RNAi can produce a null phenocopy for some genes, RNAi is by no means a substitute for genetic mutants.

2. Materials
2.1. DNA Templates for dsRNA Production

1. For cloning gene fragments into plasmid-based templates: choose a vector with the appropriate bacteriophage promoter sites (**Fig. 1**, *Constructs 1–4,6*) and appropriately placed restriction sites. Plasmid vectors are available from several suppliers of molecular biology products. pBlueScript (Stratagene) has T3 and T7 promoter sites that could be used for *Constructs 1, 3–6*. A "double T7" plasmid (L4440, generated by the Fire Lab *[3]*), (**Fig. 1**, *Construct 2*; and **Fig. 2**) is used in the feeding protocol and is available from the *Caenorhabditis* Genetics
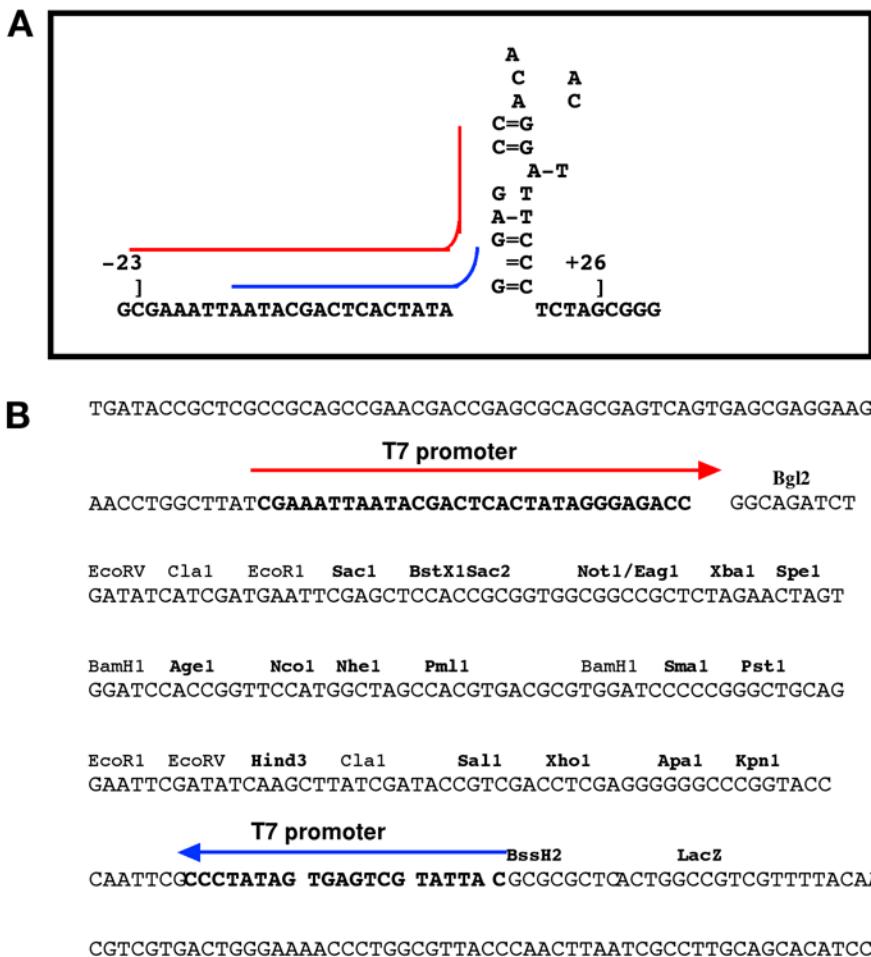
Fig. 2. T7 promoter regions used in L4440 (pPD129.36) "feeding vector" *(3)*. **(A)** Promoter region for bacteriophage T7 gene 10A (major capsid protein). Regions beneath black or gray lines are used in the L4440 vector; the corresponding sequence in **B** is similarly highlighted. **(B)** T7 promoter, restriction bank, and flanking regions in L4440; unique restriction sites bolded.

Center at the University of Minnesota (http://biosci.umn.edu/CGC/CGChome page.htm). Constructs with this plasmid backbone can also be used for in vitro transcription of dsRNA either from the intact plasmid or from a PCR-generated fragment (*see* flanking sequences in **Fig. 2**).

2. For cloning a DNA segment of the gene of interest: cDNAs are available from several sources, most notably, from the *est* database of Yuji Kohara: http://www.

ddbj.nig.ac.jp/c-elegans/html/CE_INDEX.html or http://nematode.lab.nig.ac.jp/dbest/srchbyclone.html *est*-derived plasmids harbor individual cDNA segments in a pBlueScript background (**Fig. 1**, *Construct 1*). Segments of the gene of interest can also be obtained by amplifying a coding region from genomic DNA, or by reverse transcription of an RNA sample derived from worms followed by PCR. DNA primers will be needed in the latter applications; primers designed with specific restriction sites on the ends may also be needed for proper subcloning.

3. To generate PCR fragments that can be used as transcription templates, amplify the gene of interest from genomic DNA, a cDNA clone, etc. PCR primers should be designed to include a stretch of T7 promoter sequence in the 5'-end of each primer followed by specific gene sequences. For example, in the primers: 5'-GTAATACGACTCACTATAGGxxxx…. and 5'-GCCTATAGTGAGTCGTA TTAxxx…. (where *x* represents target gene sequences), T7 sites are present in each primer, allowing dsRNA to be synthesized in one tube (*see* **Fig. 2**).

4. Reagents and supplies for cloning: restriction enzymes and buffers, agarose gel electrophoresis, supplies for DNA separation and analysis, DNA affinity purification kit, thermostable enzymes and buffers for PCR are also commercially available in kit format, primers, dNTP solutions.

5. Commercial resources: plasmids with *C. elegans* gene fragments cloned into L4440 (**Fig. 1**, *Construct 2*) are available from Geneservice Ltd. *(4)* (http://www.geneservice.co.uk/products/rnai/index.jsp). Approximately 16,757 clones are available in the "feeding" library. Also, full-length cDNA fragments in the Gateway (Invitrogen) vectors are available from Open Biosystems *(5)* (http://www.openbiosystems.com) more than 11,500 clones are in the Open Biosystems library.

2.2. Production of dsRNA by In Vitro Transcription

In vitro transcription protocols employ a simple bacteriophage RNA polymerase along with a DNA template that harbors the corresponding promoter sequences. The RNA polymerase binds to the promoter and synthesizes a copy of single-stranded RNA in a directional and strand-specific manner. PCR products as well as plasmids can be used as templates. In vitro transcription involves preparation of the template DNA (purification and restriction digestion), synthesis of RNA, annealing of sense and anti-sense strands, and lastly, analysis of the ssRNA and dsRNA products. In vitro transcription kits are available from several commercial sources.

1. Purified template DNA (plasmid or PCR product; **Fig. 1**), restriction enzymes, agarose gel electrophoresis apparatus and buffers, ultraviolet transilluminator.

2. Running buffer for RNA analysis: 0.5X TAE (20 m*M* Tris-acetate, 0.5 m*M* EDTA, 0.3 µg/mL ethidium bromide; filtered); 1% agarose gel for RNA analysis (gel is run in 0.5X TAE).

3. DNA affinity purification kit, phenol/chloroform/isoamyl alcohol (25:24:1), 100% ethanol, 3 *M* sodium acetate (pH 5.2), 70% ethanol.

4. Reagents for transcription reactions: 200 m*M* dithiothreitol; ribonucleotide solution (GTP, ATP, UTP, and CTP; each at 5 m*M*), nuclease-free H$_2$O, 10X transcription buffer (standard buffer: 400 m*M* Tris-HCl, pH 7.5, 100 m*M* NaCl, 60 m*M* MgCl$_2$, 20 m*M* spermidine *[6]*), RNase inhibitor, bacteriophage RNA polymerases (e.g., T7or T3) or a commercially available in vitro transcription kit, TE (25 m*M* Tris-HCl, 10 m*M* EDTA, pH 8.0).

5. Incubators, water baths, or PCR machine for various incubation temps.

6. Gloves.

3. Methods

RNAi experiments in *C. elegans* generally involve the use of long dsRNA molecules (as opposed to siRNAs) because worms do not display interferon responses to longer molecules. The ability to trigger RNAi using long dsRNAs is advantageous with respect to cost and to the robustness of the RNAi response—a longer dsRNA molecule is processed into many different siRNA species by *C. elegans* cells, allowing for an inherent amplification of RNAi in comparison to the use of a single siRNA sequence. Irrespective of the delivery method used, RNAi experiments rely on DNA templates for dsRNA production. Listed next are some guidelines for production of DNA templates and for in vitro transcription of dsRNA from these templates—*see also* **refs.** *6–9* for additional protocols, including preparation of genomic DNA, single-worm PCR, RNA preparation, and RT-PCR protocols that might prove useful in obtaining the target sequence.

3.1. DNA Templates for dsRNA Production

1. Select the precise region of the coding sequence of the gene that will be used as dsRNA trigger. A length of 200 bp is sufficient. It may be necessary to amplify sequences by PCR (or RT-PCR) from genomic or cDNA sources.

2. Decide upon the DNA configuration you need for your experiment (*see* **Fig. 1** and **Subheading 2.**). dsRNA prepared from a PCR-generated template can be utilized in injection and soaking delivery methods; *Construct 2* is a convenient configuration for this as it allows both strands of dsRNA to be transcribed in the same tube using T7 RNA polymerase (**Fig. 1**). For plasmid-based transcription templates (**Fig. 1**; *Constructs 1,3–6*), obtain a plasmid vector with the appropriate phage promoters, and determine the restriction sites that will be used to insert the gene of interest or *C. elegans* promoter.

3. For *Constructs 3–6* (**Fig. 1**), the final gene configuration should contain two oppositely oriented copies of the same DNA segment flanking a stuffer fragment. An inverted repeat can be cloned in two steps behind a promoter (*see* **Fig. 3**).

4. Subclone the DNA segments using standard molecular techniques (*see also* **ref.** *6*).
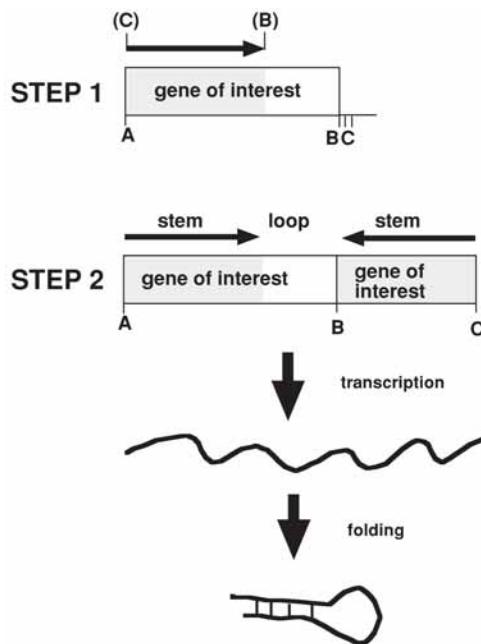
Fig. 3. A suggested cloning strategy for producing inverted repeat configurations of gene segments. A reasonable length for the "stem" segment is 100–500 bp and the "loop" or "stuffer" segment should be roughly equal to, or greater, in size to the stem for efficient cloning—if the inverted repeat sections are too close together, they will not be properly maintained in bacterial cells. This strategy uses sequences from the gene of interest as the loop segment, thereby avoiding the potential for unrelated sequences to inadvertently contribute to interference. In step 1, a large section of cDNA is cloned into restriction sites A and B of a suitable cloning vector. Primers designed with restriction sites C and B at the 5'-end will allow amplification of the front section of the gene of interest (*see* horizontal arrow in step 1). This fragment is cloned into the B and C sites present in the plasmid generated in step 1. Transcription of this inverted repeat will yield a hairpin RNA.

3.2. Production of dsRNA by In Vitro Transcription

1. Plasmid DNA should be of high quality and can be purified using a method that minimizes salt concentrations (a number of commercial kits are available). In vitro transcription kits with T7, T3, and also Sp6 RNA polymerases can also be purchased from a number of commercial sources. These provide a good yield and reproducibility. The DNA template (5–10 µg) is first linearized using a restriction enzyme at the end of the fragment (corresponding to the 3'-end of the transcribed RNA). For dual promoter plasmids, two digestions in separate tubes may

be required—one tube will be used to generate the sense strand and the other for the antisense strand. For double-promoter plasmids (**Fig. 1**, *Construct 2*), the two digested plasmids can be combined into one tube and transcribed simultaneously using one RNA polymerase. For single-promoter plasmids harboring an inverted repeat DNA sequence, the plasmid should be digested with a restriction enzyme at the end of the inverted repeat. Enzymes that leave a 5'-overhang or blunt end should be chosen because RNA polymerases may initiate transcription from a 3'-overhang. DNA generated by PCR can be used directly as a template in in vitro transcription reactions following a clean-up step. Purification of the DNA from reaction components can be easily accomplished using commercial kits or by phenol/chloroform extraction followed by ethanol precipitation.

2. Analyze a small aliquot of the digestions by agarose gel electrophoresis to confirm the plasmid DNA was completely linearized.

3. Clean up the digestions to remove enzymes and salts by purifying over a DNA affinity column (commercial kits are available) or perform phenol/chloroform extraction and ethanol precipitation.

4. Transcription kits:
   a. For commercial transcription kits, mix the reaction components and template DNA as per manufacturer's instructions. Incubate the reaction for 1–5 h at 37°C or per manufacturers instructions.
   b. If commercial kits are not available, use the following reaction. Add components in the order listed to prevent precipitation of DNA with the spermidine in the buffer (equilibrate components to room temp unless otherwise noted):
      i. 0.5–1.0 μg linearized template DNA or PCR fragment.
      ii. 1 μL of 200 m*M* dithiothreitol.
      iii. 2 μL of ribonucleotide solution.
      iv. H_2O to 16 μL.
      v. 2 μL of 10X transcription buffer.
      vi. 24 U of RNase inhibitor (stored on ice).
      vii. 15–20 U of RNA polymerase (stored on ice).
      viii. Total reaction volume is 20 μL.
      ix. Incubate the reaction for 1–5 h at 37°C.

5. Remove a 0.5-μL sample from each single-stranded reaction for analysis by agarose gel electrophoresis. Typical yields range from 0.2 to 2.0 μg/μL. It is not always necessary to anneal or to clean up a dsRNA sample before soaking or injecting.

6. Transcribed hairpin RNAs should not require an annealing step. When sense and antisense strands are synthesized separately, it sometimes helps to perform an annealing step to optimize the yield of fully dsRNA. Annealing and clean-up can be performed simultaneously by phenol/chloroform treatment of the mixed strands, followed by ethanol precipitation. DNase treatment of the sample may be performed before the phenol/chloroform step. Alternatively, the strands can be combined, heated to 65°C and slowly cooled.

7. Remove a 0.5-μL sample of the annealed RNA for analysis by agarose gel electrophoresis.

4. Notes

1. It is possible to produce a hybrid dsRNA molecule by inserting two trigger sequences into an RNA expression plasmid. Efficient RNAi for both gene targets can be observed. If one of the sequences corresponds to *rrf-3* or *eri-1 (10, 11),* endogenous inhibitors of RNAi, the efficiency of RNAi can be enhanced.
2. A more robust RNAi response using the feeding protocol can sometimes be obtained using mutant strains with enhanced RNAi activity such as strains with mutations in *rrf-3* or *eri-1.*

References

1. Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391,** 806–811.
2. Tabara, H., Grishok, A., and Mello, C. C. (1998) RNAi in *C. elegans*: soaking in the genome sequence. *Science* **282,** 430–431.
3. Timmons, L. and Fire, A. (1998) Specific interference by ingested dsRNA. *Nature* **395,** 854.
4. Kamath, R. S., Fraser, A. G., Dong, Y., et al. (2003) Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* **421,** 231–237.
5. Rual, J. F., Ceron, J., Koreth, J., et al. (2004) Toward improving *Caenorhabditis elegans* phenome mapping with an ORFeome-based RNAi library. *Genome Res.* **14,** 2162–2168.
6. Hull, D. and Timmons, L. (2004) Methods for delivery of double-stranded RNA into *Caenorhabditis elegans*. *Methods Mol. Biol.* **265,** 23–58.
7. Wood, W. B. (1988) *The Nematode* Caenorhabditis elegans*,* Cold Spring Harbor Press, Cold Spring Harbor, NY.
8. Epstein, H. F. and Shakes, D. C. (1995) Caenorhabditis elegans*: Modern Biological Analysis of an Organism,* Academic Press, New York, NY.
9. Hope, I. A. (1999) C. elegans*: A Practical Approach,* Oxford University Press Oxford, UK.
10. Simmer, F., Tijsterman, M., Parrish, S., et al. (2002) Loss of the putative RNA-directed RNA polymerase RRF-3 makes *C. elegans* hypersensitive to RNAi. *Curr. Biol.* **12,** 1317–1319.
11. Kennedy, S., Wang, D., and Ruvkun, G. (2004) A conserved siRNA-degrading RNase negatively regulates RNA interference in *C. elegans*. *Nature* **427,** 645–649.

9

Delivery Methods for RNA Interference in *C. elegans*

Lisa Timmons

Summary

This chapter describes four methods for delivery of double-stranded RNA to *Caenorhabditis elegans* (injection, feeding, soaking, and in vivo delivery), and suggests schemes that should facilitate detection of specific gene silencing.

Key Words: Double-stranded RNA; dsRNA; single-stranded RNA; ssRNA; RNAi; RNA silencing; feeding; soaking; injection; HT115; HT115(DE3); transgene; hairpin RNA; RNaseIII; transcription; functional genomics.

1. Introduction

We present four protocols that should allow efficient delivery of double-stranded RNAs (dsRNAs) to the animal: (1) injection involves preparing dsRNA by in vitro transcription followed by injection of the dsRNA into animals; (2) soaking is a forced ingestion or absorption of dsRNA by suspension of animals in concentrated dsRNA solutions; (3) feeding relies on ingestion of bacteria engineered to express dsRNA; and (4) in vivo delivery is accomplished by creating DNA constructs designed to express dsRNA from *Caenorhabditis elegans* promoters and transforming such constructs into worms to generate transgenic lines.

Each of the delivery methods has particular advantages and limitations. The relative dosage of dsRNA may account for some limitations: for injection and soaking, a wide range of dsRNA concentrations can be utilized; however, the dosage of dsRNA delivered by soaking, as well as feeding, relies on an adequate ingestion or absorption by the animal. Injection experiments are more labor-intensive than other methods and yield fewer affected progeny than feeding or transgene delivery. Libraries of feeding strains are available that can

facilitate RNA interference (RNAi)-based screening of the entire genome. In such screens, worms are fed one bacterial strain at a time. For in vivo delivery of dsRNAs from transgenes, the timing and location of silenced cells is determined by the transcriptional properties of the transgene and also the ability of RNA silencing signals to spread from the tissue of origin.

RNAi phenotypes may be difficult to reproduce. Growth temperature, developmental stage at the time of dsRNA delivery, or the presence of contaminating microorganisms can affect the efficacy. To fully assess an RNAi phenotype, it is advisable to administer dsRNA to animals of the same developmental stage, clone treated animals on individual culture plates, group plates into sets, and place sets under different growth conditions. Each animal and its progeny should be monitored for phenotype(s).

RNAi experiments can be performed in wild-type or mutant strains, provided the strains are amenable to RNAi. Strains with mutations in *rrf-3* or *eri-1* display stronger RNAi responses than wild-type; however, these strains do display developmental phenotypes such as temperature sensitive sterility (*1,2*).

Finally, please remember that although RNAi can reliably produce a null phenocopy for some genes, RNAi is by no means a substitute for genetic mutants.

2. Materials

2.1. Delivery of dsRNA by Injection

1. 0.1–3.0 μg/μL dsRNA prepared by in vitro transcription (*see* Chapter 8).
2. Microinjection equipment: inverted microscope, needle puller; Pasteur pipets, forceps, glass slides, standard borosilicate, thin wall, filamented capillary tubes, outer diameter/inner diameter within the range: 1.0/0.58 to 1.5/0.84 mm; mineral oil (heavy white oil; viscosity of 100°C: 340–360); injection pads; injection needles (*see* **Note 1**).
3. M9 medium (1 L): 3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 mL 1 *M* $MgSO_4$.
4. Binocular dissection microscope with transmitted light source.
5. OP50-Seeded nematode growth media (NGM) plates (*[3,4]* OP50 bacteria can be obtained from the *Caenorhabditis* Genetics Center (http://biosci.umn.edu/CGC/CGChomepage.htm).
6. Appropriately staged worms.

2.2. Delivery of dsRNA by Soaking

1. dsRNA (0.2-10 μg/μL) prepared by in vitro transcription (*see* Chapter 8), sterile microfuge tubes; sterile dH_2O; appropriate strain of *C. elegans*, 2X soaking buffer (M9 buffer without magnesium, 7 m*M* spermidine, 0.1% gelatin).
2. 15°C incubator, additional incubators at appropriate temperature.
3. OP50-seeded NGM plates, mineral oil.
4. Appropriate microscope for phenotypic analysis.

5. Appropriately staged *C. elegans.* Gravid adults may be bleached *(4)* and embryos recovered onto OP50 seeded plates as a means of producing lots of clean L1 larvae that will be produced 24 h after bleaching.

2.3. Delivery of dsRNA by Feeding Worms dsRNA-Expressing Bacteria

1. DNA plasmid:
    a. DNA plasmid containing the gene of interest inserted between two T7 promoter sites (*see* Chapter 8, **Fig. 1**, *Construct 2*).
    b. OR DNA plasmid containing a single T7 promoter site followed by the gene-of-interest in inverted repeat configuration (*see* Chapter 8, **Fig. 1**, *Construct 4*).
2. Competent HT115(DE3) bacterial cells *(3,5)*—HT115(DE3) is a tetracycline-resistant, RNaseIII (–) strain available from the CGC; LB broth and agar plates, 12.5 mg/mL Tetracycline (Tet), 50–100 mg/mL ampicillin (Amp) or 25 mg/mL Carbenecillin (1000X stock concentrations), 37°C shaking incubator
3. Isopropyl-β-D-thiogalactopyranoside (IPTG), stock solution = 4 mM (1000X) plates containing NGM agar supplemented with 12.5 μg/mL Tet, the antibiotic appropriate for plasmid selection (50–100 μg/mL Amp or 25 μg/mL Carb), and 0.4 mM IPTG. (Standard 60- × 15-mm Petri dishes can be used; 6-well and 24-well multiwell formats can also be used.)
4. Appropriate strain of *C. elegans*.

2.4. Delivery of dsRNA by In Vivo Transcription

1. A plasmid configured with inverted DNA repeats behind a *C. elegans* promoter (*see* Chapter 8, **Fig. 1**, *Construct 5* or *6*).
2. A plasmid that can be used as a transformation marker that will express a dominant *C. elegans* trait that will not interfere with subsequent phenotypic analysis (green fluorescent protein-expressing plasmids, pRF4 plasmid with dominant "roller" mutation in *rol-6,* and others)*.
3. Microinjection equipment: inverted microscope, needle puller, Pasteur pipets, forceps, glass slides, standard borosilicate, thin wall, filamented capillary tubes, outer diameter/inner diameter within the range: 1.0/0.58 to 1.5/0.84 mm, mineral oil (heavy white oil, viscosity of 100°C: 340–360), injection pads, injection needles *(3,4)*.
4. M9 medium (1 L): 3 g KH$_2$PO$_4$, 6 g Na$_2$HPO$_4$, 5 g NaCl, 1 mL 1 M MgSO$_4$.
5. Binocular dissection microscope with transmitted light source.
6. OP50-Seeded NGM plates *(3,4)*.
7. Appropriately staged worms.

3. Methods
3.1. Delivery of dsRNA by Injection

An RNAi phenocopy can be induced in *C. elegans* by injecting dsRNA into the animals *(6)*. It is possible to observe phenotypes in the injected animals and in treated progeny. Essential genes can be studied by injecting into younger animals, then scoring for defects as the treated animal ages.

1. Inject the dsRNA solution prepared by in vitro transcription (*see* Chapter 8) into the gonad of young adult hermaphrodites. (Injection into the gonad yields the greatest number of affected progeny; however, injecting dsRNA into the intestine or body cavity is also successful in producing an RNAi phenocopy in progeny. Younger larvae can also be injected to allow for observation of RNAi in treated animals [*see* **Note 1**]).
2. Add M9 recovery buffer to injected animals on pad.
3. Carefully transfer injected animals onto OP50-seeded NGM plates using a pipet. Transfer injected animals to fresh plates daily, and score injected animals and progeny for phenotypes. Label the plates so that all progeny of an injected individual can be monitored as a group (*see* **Note 2**).
4. Monitor each batch of progeny for phenotypes. The first batch of progeny may lack phenotypes because as developing oocytes or embryos, they may have been too developmentally progressed at the time of dsRNA delivery. Later batches of progeny may not be affected as the dsRNA may be degraded or may become limiting.
5. Compare the phenotypic distributions between the sets of progeny and between sets of injections. It is wise to perform several sets of injections, culturing each set of worms postinjection under different temperature or other growth conditions.

3.2. Delivery of dsRNA by Soaking

An RNAi phenotype can be induced in *C. elegans* by soaking the worms in a concentrated solution of dsRNA made by in vitro transcription *(7,8)*.

1. Set up varying dilutions of dsRNA (0.2–5.0 µg/µL) in microfuge tubes in 5 µL volume (minimum). Dilutions can be made using sterile water or 2X soaking buffer (*see* **Note 3**). Tubes are capped to prevent evaporation.
2. Add 10–20 worms of the appropriate strain and developmental stage to the diluted dsRNA. Bacteria can be removed from the surface of the worms by first picking them onto unseeded OP50 plates, allowing them to crawl, then placing them into the soaking solution. Animals at L4 stage will produce affected embryos; animals at L1 stage may display RNAi phenocopies as they age.
3. Incubate overnight at 15°C. Longer incubations may result in better phenocopy production. Incubation at 20°C can also produce good results; however, incubations at 25°C can prove problematic.
4. After incubation, carefully transfer worms from the tube to a seeded NGM plate using a 200-µL pipet tip set at 20 µL. Carefully rinse the pipet tip and the tube with a small amount of sterile dH_2O and mineral oil to ensure all worms have been transferred (*see* **Note 2**).
5. After a few hours, clone individual soaked worms to individual OP50-seeded NGM plates.
6. After 24 h, or when F_1 embryos are observed, the soaked worms should be transferred to a fresh plate. This is done to flush out the first batch of F_1 progeny that

may have been present within the adult animal at the time of soaking, and therefore may not show a phenotype. Subsequent transfers of soaked animals on following days is also advisable because the RNAi effects do wear off and phenotypes may not be observed in later progeny.

7. Periodically monitor the soaked animals and F_1 progeny for phenotypes.

3.3. Delivery of dsRNA by Feeding

The feeding protocol requires a plasmid template with DNA sequences corresponding to the dsRNA trigger inserted between two, oppositely configured bacteriophage T7 promoter sites (*see* Chapter 8, **Fig. 1**, *Construct 2*; and **refs. *3,5,9* and *10***). (Such plasmids are available from commercial sources; *see* Chapter 8.) Alternatively, the target gene can be configured as an inverted repeat and inserted behind a single T7 promoter site (*see* Chapter 8, **Fig. 1**, *Construct 4*). The plasmid is transformed into HT115(DE3) host cells (*see* **Note 4**), and dsRNA production is maintained on NGM plates supplemented with antibiotics and IPTG. Worms are placed directly on such plates and phenocopies are monitored in the presence of food. The protocols below require ampicillin or carbenicillin selection for maintenance of plasmids in the bacterial strain. Use sterile techniques for all the protocols listed next.

1. Inoculate a 2-mL 2X YT culture containing 12.5 µg/mL Tet and 50–100 µg/mL Amp with a single colony of HT115(DE3) + plasmid and incubate overnight at 37°C with shaking at 225 rpm.
2. Dilute the culture more than 100-fold and continue to grow until culture reaches $OD_{600} = 0.4$–0.8.
3. Add IPTG to the culture to a final concentration of 0.4 m*M* and incubate with shaking (225 rpm) for an additional 1 h at 37°C. This induces transcription by T7 RNA polymerase within the cells.
4. Supplement the culture with additional 50 µg/mL Amp, 12.5 µg/mL Tet, and 0.4 m*M* IPTG.
5. Directly seed the induced cells onto NGM plates supplemented with 50–100 µg/mL Amp (or 25 µg/µL Carb), 12.5 µg/mL Tet, and 0.4 m*M* IPTG. Allow cell lawns to dry at room temp overnight.
   Alternatively *(9)*,
   a. Inoculate a 2-mL 2X YT culture containing 12.5 µg/mL Tet and 50–100 µg/mL Amp with a single colony of HT115(DE3) + plasmid and incubate 6–8 h at 37°C.
   b. Directly seed the cells onto NGM plates supplemented with 25 µg/µL Carbenicillin and 4 m*M* IPTG.
   c. Proceed to **step 6**.
6. Transfer worms to plates using a worm pick to transfer individuals or a metal spatula to transfer a small agar chunk with more worms (*see* **Note 5**).
7. Monitor phenotypes in the transferred animals and in their progeny (*see* **Note 6**).

3.4. Delivery of dsRNA by In Vivo Transcription

1. Plasmid DNAs are injected into the gonad of animals to generate transgenic lines (*4*).
2. Each set of animals is recovered onto NGM/OP50 plates. The progeny that exhibit a phenotype corresponding to the transformation marker are cloned onto separate plates. These F_1 progeny are monitored for the presence of the marker phenotype—not all F_1s will give rise to lines. Those plates producing F_2 animals with the marker phenotype are maintained as separate lines.
3. Each transformed line should be examined for a RNAi phenocopy using different culture conditions (different temperatures, for example).
4. The efficacy of RNA silencing can be monitored by performing *in situ* RNA hybridizations or protein immunolocalization if an antibody to the target protein is available.

4. Notes

1. A number of different protocols are published that describe microinjection in *C. elegans* in detail (*3,4,6,11*).
2. For efficient worm recovery from pipet tips, we first place a drop of mineral oil and a drop of M9 on a recovery plate. Worms are placed into a drop of M9 on an NGM plate, using the same pipet tip to transfer all worms. Worms are counted before and after transfer. If worms are stuck to the pipet tip, they are removed by pipeting mineral oil from the plate slowly up and down the pipet tip and expelling the mineral oil onto the plate, followed by pipeting similarly with more M9. The mineral oil will release the worms from the plastic tip surface, and the M9 will help wash the worms and mineral oil from the tip.
3. Animals should also be soaked in control solutions that are as consistent with the experimetal solution as possible. These controls can help identify toxic components in the dilution buffers or in in vitro transcription buffers.
4. The feeding protocol works best when the HT115(DE3) cells are freshly transformed. Storage of expression strains at 4°C on LB plates often results in a loss of competency for dsRNA production. Standard methods for maintaining, freezing, and rendering cells competent by $CaCl_2$ can be used with this strain of bacteria (*3*). Plasmid DNA can be isolated from HT115 cells using standard mini-prep procedures; however, this strain has higher nuclease activity than standard cloning strains, so DNA yields may be low.
5. It is best to use freshly seeded plates as opposed to plates that have been stored. RNAi phenotypes are usually observable within 16 h and 3 d, depending on the target gene, quality of food, and incubation temperature. Plates that are 60×15 mm contain sufficient quantities of bacteria to support growth of the worms for a few generations; subsequent generations can be transferred to fresh plates as food depletion can diminish the RNAi phenotype.
6. Generally, only one strain of bacteria is fed to worms, as RNAi effectiveness decreases with increasing food complexity. The use of *C. elegans rrf-1* or *eri-1*

mutants in double feeding experiments may improve RNAi effectiveness for each target. The feeding protocol can be adapted to liquid culture.

Acknowledgments

References

1. Simmer, F., Tijsterman, M., Parrish, S., et al. (2002) Loss of the putative RNA-directed RNA polymerase RRF-3 makes *C. elegans* hypersensitive to RNAi. *Curr. Biol.* **12,** 1317–1319.
2. Kennedy, S., Wang, D., and Ruvkun, G. (2004) A conserved siRNA-degrading RNase negatively regulates RNA interference in *C. elegans*. *Nature* **427,** 645–649.
3. Kamath, R. S., Martinez-Campos, M., Zipperlen, P., Fraser, A. G., and Ahringer, J. (2001) Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*. *Genome Biol.* **2,** RESEARCH0002.
4. Hope, I. A. (1999) C. elegans*: A Practical Approach,* Oxford University Press, Oxford, UK.
5. Timmons, L., Court, D. L., and Fire, A. (2001) Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. *Gene* **263,** 103–112.
6. Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391,** 806–811.
7. Tabara, H., Grishok, A., and Mello, C. C. (1998) RNAi in *C. elegans*: soaking in the genome sequence. *Science* **282,** 430–431.
8. Maeda, I., Kohara, Y., Yamamoto, M., and Sugimoto, A. (2001) Large-scale analysis of gene function in *Caenorhabditis elegans* by high-throughput RNAi. *Curr. Biol.* **11,** 171–176.
9. Timmons, L. and Fire, A. (1998) Specific interference by ingested dsRNA. *Nature* **395,** 854.
10. Hull, D. and Timmons, L. (2004) Methods for delivery of double-stranded RNA into *Caenorhabditis elegans*. *Methods Mol. Biol.* **265,** 23–584.
11. Epstein, H. F. and Shakes, D. C. (1995) Caenorhabditis elegans*: Modern Biological Analysis of an Organism,* Academic Press, New York, NY.

10

Functional Genomic Approaches in *C. elegans*

Todd Lamitina

Summary

The nematode *Caenorhabditis elegans* is an extraordinarily powerful model organism for the application of functional genomic approaches. Two such approaches, whole genome microarray analysis and genome-wide RNA interference (RNAi)-mediated phenotypic screening, are highly advanced and can be used by virtually any laboratory to study biological processes of interest. Using studies of the osmotic stress response in *C. elegans* as an example, this chapter describes methods for conducting whole genome microarray experiments and for carrying out genome-wide reverse-genetic screens using a commercially available *C. elegans* bacterial RNAi feeding library. Both approaches are complimentary and can be used to rapidly gain genome-wide insights into the genes and gene networks controlling specific physiological processes.

Key Words: RNA interference; microarray; genome-wide; screening; *Caenorhabditis elegans*.

1. Introduction

Because of its highly accurate, complete, and well-annotated genome sequence, *Caenorhabditis elegans* is an ideal model system for the application of functional genomic approaches to address fundamental biological questions. Although several types functional genomic approaches are possible in *C. elegans*, this chapter will focus exclusively on methods for performing whole genome microarray analysis and genome-wide RNAi screening. Both approaches are complementary and have been used with great success to begin defining genes and gene networks involved in numerous biological processes, such as fat regulation (*1*), aging (*2,3*) DNA damage and repair (*4*), and RNA interference (*5*). Whole genome gene expression profiling allows the rapid identification of genes that are differentially expressed during development or environmental adaptation. Promoter regions from differentially expressed genes can then be used to control

the expression of reporter proteins (such as green fluorescent protein [GFP]) in living worms. The regulated expression of such reporter transgenes can often times be used as a phenotypic tool to identify the genes and gene networks controlling the particular process under investigation using genome-wide RNA-mediated interference (RNAi) screening. Studies of the osmotic stress response will be used to illustrate the methods utilized for gene expression profiling and whole genome RNAi screening in *C. elegans*.

2. Materials

2.1. Microarray Materials

1. *C. elegans* strains (freely available from the *C. elegans* Genetics Stock Center).
2. 15-cm Enriched peptone agarose growth plates seeded with a lawn of *Escherichia coli* strain NA22.
3. Swinging bucket centrifuge.
4. Spectrophotometer.
5. M9 solution.
6. TRIZOL reagent (Invitrogen, Carlsbad, CA).
7. Oligo dT Cellulose (Ambion, Austin, TX).
8. Polyprep columns (Bio-Rad, Hercules, CA).
9. Chloroform.
10. Isopropanol.
11. Diethylpyrocarbonate-treated water.
12. 50- and 15-mL polypropylene conical tubes.
13. Liquid nitrogen.

2.2. Whole-Genome RNAi Screening Materials

1. *C. elegans* bacterial RNAi feeding library (MRC GeneService, Cambridge, UK).
2. 384- and 96-pin replicating and alignment tools (Nunc, Rochester, NY).
3. Eight-channel pipettors (2–20 µL and 20–200 µL).
4. 384-, 96-, and 24-multiwell plates.
5. Omnitrays (Nunc, Rochester, NY).
6. 10-cm Petri dishes.
7. 6-L Erlenmeyer flasks.
8. Qfill2 microfluidic dispenser (Genetix, Boston, MA).
9. Sterile media dispenser (Pourboy III, Tritech Research, Los Angeles, CA).
10. Isopropyl-β-D-thiogalactopyranoside(IPTG; Fisher Biotech Grade).
11. Tetracycline HCl.
12. 16 and 37°C incubators.
13. Fluorescence-equipped dissecting microscope (Zeiss M2Bio).

3. Methods

The methods described outline the steps necessary for (1) designing a microarray experiment and purifying mRNA from *C. elegans* for microarray

analysis, (2) replicating and storing *C. elegans* bacterial RNAi feeding library, and (3) performing a whole-genome RNAi screen.

3.1. Microarray Experimental Design and mRNA Purification

The purification of mRNA from *C. elegans* is described in **Subheadings 3.1.1.–3.1.4.** This includes methods for (1) designing a microarray experiment, (2) growing large scale cultures of *C. elegans,* (3) purifying total RNA, (4) and purifying polyA mRNA. Whole-genome microarray chips for *C. elegans* are available from multiple vendors (Affymetrix, www.affymetrix.com; Agilent Technologies, www.agilent.com; Washington University Genome Sequencing Center, www.genome.wustl.edu). Description of the methods for sample labeling, hybridization, scanning, and data analysis using each of these platforms are beyond the scope of this chapter. However, RNA purified using methods outlined in this chapter is compatible with each of the previously mentioned microarray chips. All experiments should be performed using the same chip set, as recent studies suggest significant variability between data obtained using different microarray platforms (*6,7*). Users are encouraged to perform labeling and hybridization reactions and data analysis through institutional microarray core facilities. If such facilities are unavailable, several commercial vendors offer this service.

3.1.1. Experimental Design

To analyze changes in gene expression induced by hypertonic stress, we performed a normalized time course experiment. In this approach, synchronized young adults were exposed to 175 m*M* NaCl for 0–12 h, and worms were harvested each hour for RNA isolation. Each time point was replicated three to five times in separate experiments to allow statistical analysis of the data. In order to compare data between time points, a reference RNA sample was generated from a population of mixed stage wild-type animals. Each time point was then compared with the same reference RNA sample to produce relative changes in gene expression. To resolve changes in gene expression caused be exposure to hypertonic stress over time, we subtracted the $t = 0$ data from each of the other time points. We then identified differentially expressed genes by performing a one-way analysis of variance using a Bonferroni correction. Genes with corrected *p* values of less than 0.05 were considered differentially expressed (*see* **Note 1**).

3.1.2. Large-Scale Worm Culture

Currently, large quantities of worms are needed to generate enough polyA RNA for microarray experiments (*see* **Note 2**). For studies of the osmotic stress response, worms were grown on 15-cm plates containing an enriched peptone

media and a dense lawn of *E. coli* strain NA22. Using this approach, suitable quantities of RNA were isolated from three to five growth plates per condition.

1. Isolate eggs using the hypochlorite method and synchronize the strain by allowing the eggs to hatch in the absence of food overnight.
2. Wash the synchronized larvae twice in isotonic M9.
3. Place approx 25 μL of synchronized L1 stage animals onto twelve 15-cm NA22 plates.
4. Culture worms at 25°C for 48 h to reach young adulthood. If lower temperatures are to be used (16 or 20°C), the number of synchronized L1s applied to each plate should be reduced to prevent the animals from starving over longer growth periods.
5. Wash adults off the growth plates and pellet by centrifuging at 700*g* for 1–2 min (should produce a worm pellet of 7–9 mL).
6. Place 500 μL of adults onto 4–15 cm NA22 plates containing 175 m*M* NaCl (four to five time points were processed in a single experiment).
7. At appropriate time intervals, wash the worms off plates using 10 mL of osmotically adjusted M9 (175 m*M* NaCl) per plate. Pool all of the washes into a single 50-mL conical tube and wash three to five times with 40–50 mL of adjusted M9 to eliminate as much bacteria as possible.
8. Transfer the washed worm pellet to a 15-mL polypropylene conical tube (*see* **Note 3**).
9. Add 4 mL of Trizol reagent per 1 mL of packed worms and vortex vigorously for 1–2 min.
10. Flash freeze in liquid nitrogen and thaw at 37°C; repeat this freeze–thaw step once.
11. Store the frozen worms at –80°C until RNA can be isolated.

3.1.3. Purification of Total RNA

1. Thaw the samples and add 2 mL of Trizol/mL of worms.
2. Shake for the tube by hand for 15 s.
3. Incubate the tubes at room temperature for 3 min.
4. Spin at 2600*g* for 1 h at 4°C.
5. Remove the upper aqueous phase to a new 15-mL polypropylene conical tube.
6. Add an equal volume of isopropanol, mix, and sit at room temperature for 10 min.
7. Spin at 2600*g* for 30 min at 4°C to pellet the total RNA.
8. Wash the RNA pellet with 8 mL of 75% EtOH.
9. Spin at 2600*g* for 30 min at 4°C to pellet the total RNA.
10. Remove as much EtOH as possible and air-dry the pellet for 10 min (careful not to dry for too long, a completely dry RNA pellet is impossible to resolublize).
11. Resuspend RNA in 1 mL of DEPC-treated water (*8*).
12. Heat at 55°C for 10 min and triturate the sample to resuspend.
13. Check the A260 and 260/280 ratios using a UV spectrophotomer. The 260/280 ratio for clean RNA should be close to 2.0.

3.1.4. Purification of PolyA RNA

For studies of the osmotic stress response, we utilized cDNA microarrays produced at the Stanford Functional Genomic Facility, whose protocols required the use of once selected polyA mRNA. Typically, polyA RNA represents about 1–3% of the total RNA. Therefore, milligram quantities of total RNA are required if microgram quantities of polyA mRNA are needed.

1. Resuspend 1 g of oligo dT cellulose in 10 mL of 1X NETS (0.1 *M* NaCl, 0.01 *M* Tris-HCl, pH 7.4, 0.01 *M* EDTA) in a 15-mL conical tube.
2. Wash the cellulose three times with 10 mL of 1X NETS
3. Resuspend the cellulose in 10 mL of 2X NETS (final volume should be 13 mL).
4. Dilute 1–2 mg of total RNA to 1 mL with 10 m*M* Tris-HCl, pH 7.4.
5. Mix the RNA solution with 1 mL of resin in a 2-mL Poly-prep column that is sealed on the bottom.
6. Bind RNA to oligo dT on a nutator for 1 h at room temperature.
7. Break off the bottom of the column and place it in a 15-mL conical tube.
8. Allow the column to drain by gravity flow (little or no flow indicates an over-loaded or clogged column). Save the column flow through for subsequent gel analysis.
9. Wash the column twice with 0.7 mL of 1X NETS. Pool and save these washes.
10. Wash the column three times with 0.7 mL 1X NETS. Pool and save these washes.
11. Elute mRNA with two applications of 0.7 mL 10 m*M* Tris-HCl, pH 7.4 that has been heated to 70°C. Store the eluted mRNA on ice.
12. Using standard gel electrophoresis methods, analyze 5 µL of the flow through, washes, and elution on a 1% TAE agarose gel containing ethidium bromide. The flow through and first wash pool should contain the majority of ribosomal RNA. Wash three should have little rRNA. The elution should have some rRNA bands in addition to a smear, which represent the mRNA.
13. Divide the mRNA fraction into two 1.5-mL tubes and extract twice with chloroform to eliminate cellulose.
14. Precipitate the mRNA by adding concentrated sodium acetate to a final concentration of 0.3 *M*. Add 1 vol of isopropanol and store at –20°C for 1 h.
15. Spin at 15,000*g* for 30 min at 4°C.
16. Wash the mRNA pellet with 75% EtOH and spin at 15,000*g* for 15 min at 4°C.
17. Check the A260, and 260/280 ratio on a UV spectrophotometer. Clean RNA solutions should have a 260/280 ratio of 2.0.
18. Store the mRNA at –80°C until microarray labeling and hybridizations are to be performed.

3.2. Handling of Bacteria From the C. elegans *RNAi Feeding Library*

Gene knockdown via dsRNA mediated interference, or RNAi, is extraordinarily efficient in *C. elegans*. To generate this knockdown effect, worms can simply be fed bacteria designed to produce dsRNA homologous to a single predicted gene in the *C. elegans* genome *(9,10)*. In these bacteria, an ampicillin-
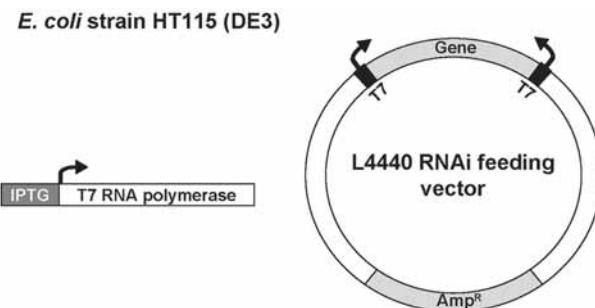
Fig. 1. Schematic representation of the genotype of double-stranded RNA (dsRNA) producing bacteria. Strain HT115(DE3) contains an isopropyl-β-ᴅ-thiogalactopyranoside (IPTG)-inducible T7 RNA polymerase within an integrated Tn10 transposon that confers tetracycline resistance to bacteria. An ampicillin-resistance plasmid containing genomic DNA homologous to a single *C. elegans* gene is flanked by two T7 RNA polymerase binding sites. When exposed to IPTG, the bacteria express T7 polymerase, which results in the production of dsRNA homologous to the DNA inserted between the T7 polymerase sites. When worms eat these bacteria, they lyse and release dsRNA. Released dsRNA is absorbed thorough the gut where it acts to silence gene expression. In a poorly understood process, this silencing effect is rapidly spread throughout the organism to silence gene expression in other tissues.

resistant plasmid contains gene-specific DNA flanked by two T7 RNA polymerase promoters. These bacteria also harbor an integrated, tetracycline-selectable transposon construct containing an IPTG-inducible T7 RNA polymerase. Therefore, bacteria only produce dsRNA in the presence of IPTG (**Fig. 1**).

A renewable bacterial library containing 16,757 bacterial strains, each producing dsRNA homologous to a single predicted gene in the *C. elegans* genome, is commercially available (www.hgmp.mrc.ac.uk/geneservice/index.shtml). Methods for replicating and utilizing this library are described in **Subheadings 3.2.1.** and **3.2.2.** This includes methods for replicating and storing the library and manipulating the library for a large-scale genome wide RNAi screen.

3.2.1. Replicating and Storing the C. elegans RNAi Feeding Library

The RNAi bacterial feeding library contains approx 16,757 bacterial strains, representing about 87% of the predicted *C. elegans* genome. This library is supplied in 55- to 384-well plates, each well containing a single bacterial clone producing dsRNA targeting one *C. elegans* gene (*see* **Note 4**). To prevent contamination of the library, at least one copy should be created and used as the working copy. The original copy should be stored in a separate location and only used in emergencies.

1. Distribute 75 μL of sterile LB media containing 25 μg/mL carbenicillin and 8% glycerol into each well of 55–384 well plates using a microfluidic media dispensor. Label each new plate to correspond with the master plates (i.e., Chromosome I, plate 1, Chromosome I, plate 2, and others).
2. Thaw each 384-well master plate to room temperature and place it within the pin replicator alignment tool. Place a 384-well plate containing media only into a second pin alignment tool.
3. Sterilize the 384-pin tool by submerging the pins in bleach, washing the pins twice in ddH₂0, once in 100% EtOH followed by flame sterilization (pipet tip box lids can be used to hold solutions; use *extreme* caution when working with EtOH near a flame).
4. Allow the pin tool to cool for approx 5 s, and then insert the pins into the master plate using the pin alignment tool. Inoculate the copy plate by inserting the pins into the second clean plate.
5. Seal the master plates with parafilm and place them back at –80°C.
6. Grow the copy plates overnight at 37°C and then seal them with Parafilm. Store the copy plates at –80°C in a separate location from the master plates (in case of catastrophic freezer malfunction).

3.2.2. Expansion of the RNAi Library
From 384-Well Plates to 96-Colony Omnitrays

Performing a genome wide RNAi screen requires expansion of the frozen bacterial RNAi library from a 384 format to a 96-colony Omnitray format to facilitate physical manipulation of the bacteria (*see* **Note 5**). In some cases, bacteria may also need to be streaked to single colonies. For example, we found that for studies of the osmotic stress response, the strongest RNAi-induced phenotypes were observed when cultures were initiated from single colonies as compared with cultures initiated from large bacterial inoculums or directly from frozen glycerol stocks. The bacteria are cultured in 96-well plates before being spotted onto 24-well nematode growth media (NGM) RNAi plates (NGM plates *[11]* containing 25 μg/mL carbenicillin and 1 m*M* IPTG). NGM RNAi plates can be poured in very large batches using a sterile media dispenser; 5 L should produce approximately seventy-five 24-well plates, which is enough to screen 1800 genes.

1. Thaw a 384-well copy plate and place it into the replicator pin alignment tool.
2. Place an omnitray containing 40 mL of LB agar containing 25 μg/mL carbenicillin and 12.5 μg/mL of tetracycline into a second pin alignment tool.
3. Sterilize the 96-pin tool as described in **Subheading 3.2.1.**, **step 3**.
4. Place the 96-pin tool into section "A" of the 384-well plate.
5. Allow the 96-pin tool to rest on top of the LB agar containing Omnitray, transferring a small amount of bacteria culture. Do not place pressure on the pin tool, as this will force the pins into the agar.
6. Sterilize the pin tool and repeat **steps 4** and **5** for sections B, C, and D of the 384-well plate, resulting in four Omnitrays/384-well.
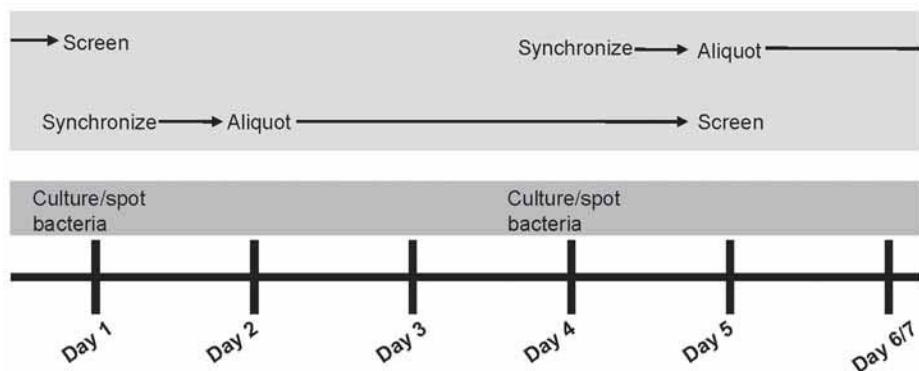
Fig. 2. Schedule for performing a feeding-based genome-wide RNA interference (RNAi) screen. A strategy for performing two RNAi screens per week is shown. Worm preparation and culture are described in **Subheading 3.3.1.**, and methods for bacterial preparation and culture are described in **Subheading 3.2.2.2.** During days 2, 3, 6, and 7, other screen tasks should be performed, such as logging data, streaking bacteria, pouring plates, and others.

7. Place the Omnitrays at 37°C overnight and then store the plates containing bacterial inoculums at 4°C.

3.2.3. Culture of RNAi Bacteria for Genome-Wide RNAi Screening

1. Streak each bacterial colony onto one quadrant of a 10-cm LB agar plate (four bacterial strains per plate) containing 25 µg/mL carbenicillin and 12.5 µg/mL of tetracycline using standard microbiological procedures *(8)*. Grow the colonies at 37°C overnight.
2. Using an 8-channel pipet, add 100 µL of LB media containing 25 µg/mL carbenicillin to each well of a 96-well plate. Label these plates to correspond to the Omnitray plate number (i.e., Chromosome I, Plate 1A, 1B, 1C, and others).
3. Pick a single colony from location A1 on the Omnitray into well A1 of the 96-well plate. Repeat for each well.
4. Grow the 96-well cultures for 4–8 h at 37°C (shaking is not necessary).
5. Spot 20–40 µL of bacterial culture per well onto a 24-well NGM RNAi plate and allow the cultures to dry in a sterile flow hood for 10–20 min.
6. Incubate the plates overnight at room temperature to allow the bacteria to produce dsRNA. Plates containing RNAi bacteria should be stored at 4°C and used within 1–2 wk.

3.3. Genome-Wide RNAi Screening

Performing a genome-wide RNAi screen requires the coordination of the bacterial methods described in **Subheading 3.2.** with worm culture methods described in **Subheading 3.3.** (**Fig. 2**). If possible, screens should be started

using hypochlorite synchronized L1 stage animals to prevent bacterial or fungal contamination. Animals are placed on RNAi growth plates containing 25 μg/mL carbenicillin and 1 m*M* IPTG (*see* **Note 6**). After 72 h at 16°C, young adult worms are screened for a visible phenotype. Alternatively, animals can be placed on RNAi growth plates for 72 h, gravid adults transferred to new RNAi growth plates, and their progeny screened for a visible phenotype after another 72 h of growth. However, the additional manipulation and culture time required in this approach can easily double or triple the time required to perform the screen.

Development of a simple, reproducible phenotypic assay is absolutely essential to a successful genome-wide RNAi screen. The results of whole-genome microarray analyses can frequently provide valuable tools for developing such assays. For example, in previous studies of the osmotic stress response, we showed that *C. elegans* accumulates glycerol as an organic osmolyte in response to hypertonic stress *(12)*. Using the results of whole-genome microarray analyses, we found that the glycerol biosynthetic enzyme F47G4.3 was transcriptionally upregulated approx 15-fold within 1 h of exposure to hypertonic stress (Lamitina and Strange, unpublished observations). Using transgenic approaches, we created animals expressing the GFP under the control of the F47G4.3 promoter ($P_{F47G4.3}$::*GFP [13]*). The transgene was then integrated into the genome using standard UV mutagenesis procedures. Under isotonic culture conditions, $P_{F47G4.3}$::*GFP* transgenic animals fail to express detectable levels of GFP when viewed under a fluorescence-equipped dissecting microscope. However, exposure to hypertonic stress causes a rapid and easily visible increase in GFP levels in 100% of the animals. Therefore, animals carrying this reporter transgene provide a simple and convenient assay with which to identify genes that regulate osmosensitive gene expression.

We performed a genome-wide RNAi screen using the bacterial feeding library to identify genes that normally act to inhibit the signaling pathways controlling $P_{F47G4.3}$::*GFP* expression. Inhibition of these genes by RNAi gives rise to animals that inappropriately express GFP under isotonic conditions. Using the methods described in **Subheadings 3.2.** and **3.3.**, two people were able to screen the RNAi feeding library in approx 5 mo. At peak levels, approx 1600 genes per week could be screened (*see* **Note 7**). For each round of screening, controls were used to indicate the effectiveness of RNAi (i.e., genes that produce lethal, sterile, or other easily detectable phenotypes) and the robustness of the GFP assay (i.e., genes that are known to activate $P_{F47G4.3}$::*GFP*; *see* **Note 8**). Bacterial clones that produce "hits" in the primary screen were rescreened an additional four times (using four different bacterial colonies) to confirm the result. For bonafide "hits," most animals should express GFP or exhibit the appropriate visible phenotype.

3.3.1. Performing a Genome-Wide RNAi Screen

1. Isolate eggs from $P_{F47G4.3}::GFP$ animals using the hypochlorite method. Synchronize the eggs by allowing them to hatch on a clean NGM plate.
2. Wash the synchronized $P_{F47G4.3}::GFP$ L1s twice in sterile M9.
3. Dilute the L1s to approx 30 animals/10 μL and distribute 10 μL per well to 24 wells spotted with dsRNA-producing bacteria (the use of multichannel pipettors to distribute worms onto the RNAi plates greatly facilitates this step). Allow the liquid to evaporate in a sterile flow hood for approx 5 min.
4. Incubate the worms at 16°C for 72 h until they reach young adulthood.
5. Visually score the animals for GFP expression under a fluorescence-equipped dissecting microscope. To be considered a "hit," the majority of animals in a well should exhibit the mutant phenotype.
6. Confirm the results of the initial screen by rescreening at least four independent bacteria colonies for each gene.

4. Notes

1. Utilizing typical measurements of statistical significance ($p < 0.05$) would result in an unacceptable number of false-positives among the genes identified as differentially regulated (for a 17,000 gene *C. elegans* chip, 850 genes would be show up as false-positives using a $p < 0.05$ cut off, even if no genes were actually differentially expressed). To circumvent these problems, more rigid statistical criteria are needed. For studies of the osmotic stress response, we performed a one-way analysis of variance using Bonferroni-corrected p values (as implemented in the microarray analysis program Genetraffic [Stratagene, La Jolla, CA]). Free microarray analysis tools are also available, such as the Microsoft Excel add-in significance analysis of microarrays (SAM; www-stat.stanford.edu/~tibs/SAM/).

   Another experimental design for microarray analysis involves single pair comparisons without the use of a reference RNA sample *(14,15)*. In this approach, RNA from two mutant strains (gain-of-function vs loss of function or mutants with opposing phenotypes) are directly compared and significance is calculated using the *t*-test.
2. More recent microarray platforms only require the use of total RNA for probe labeling and hybridization. In such cases, the number of worms required to generate acceptable quantities of RNA can be reduced by as much as 50- to 100-fold.
3. Only use polypropylene tubes, as other types of plastic will not withstand the subsequent freeze–thaw cycles in liquid nitrogen.
4. The clones contained in the bacterial RNAi library have not been confirmed by sequencing or other methods. As a result, some wells that should contain specific bacterial clones are either empty (no bacteria) or contain the incorrect clone (either an empty plasmid vector or the wrong insert). For example, 1145/4812 bacterial clones from chromosome 5 are known to be missing (24%). For the other five linkage groups, these mistakes affect 3–5% of the library.

5. The bacterial RNAi library was initially constructed in 96-well plates and then compressed into 384-well plates for distribution. Screening the library in the 96-well format makes recording screening results much easier because the Microsoft Excel spreadsheet containing clone information is also arrayed in the same 96-well format.

6. Others have performed genome-wide RNAi screens using liquid culture methods *(4,16)*. Using this method, genome-wide screens can be performed in as little as 4–6 wk. However, the physiological characteristics of worms grown in liquid vary dramatically from worms grown on agar plates containing lawns of bacteria. For example, worms exposed to hypertonic stress under plate culture conditions accumulate large amounts of the organic osmolyte glycerol *(12)*, whereas worms exposed to hypertonic stress in liquid culture do not accumulate glycerol (Lamitina and Strange, unpublished observations). Therefore, liquid culture based RNAi assays are not suitable for studies of the osmotic stress response in *C. elegans*.

7. The time required to screen the RNAi feeding library is heavily dependent on the endpoint assay. For studies of the osmotic stress response, synchronized L1 stage transgenic animals were pipetted onto RNAi plates and examined for the GFP expression phenotype after 3 d. However, the time required to screen the library can easily increase to a year or more if physical manipulation of the worms or more detailed phenotypic examination is required.

8. In *C. elegans*, transgene expression can be epigenetically silenced *(17)*. When using animals expressing GFP in a RNAi screen, great care should be taken to ensure that the transgene is performing appropriately. Several copies of the starting strain should be frozen before starting the screen and thawed if problem arise.

References

1. Ashrafi, K., Chang, F. Y., Watts, J. L., et al. (2003) Genome-wide RNAi analysis of *Caenorhabditis elegans* fat regulatory genes. *Nature* **421,** 268–272.
2. Lund, J., Tedesco, P., Duke, K., Wang, J., Kim, S. K., and Johnson, T. E. (2002) Transcriptional profile of aging in *C. elegans*. *Curr. Biol.* **12,** 1566–1573.
3. Murphy, C. T., McCarroll, S. A., Bargmann, C. I., et al. (2003) Genes that act downstream of DAF-16 to influence the lifespan of *Caenorhabditis elegans*. *Nature* **424,** 277–283.
4. van Haaften, G., Vastenhouw, N. L., Nollen, E. A., Plasterk, R. H., and Tijsterman, M. (2004) Gene interactions in the DNA damage-response pathway identified by genome-wide RNA-interference analysis of synthetic lethality. *Proc. Natl. Acad. Sci. USA* **101,** 12,992–12,996.
5. Kim, J. K., Gabel, H. W., Kamath, R. S., et al. (2005) Functional genomic analysis of RNA interference in *C. elegans*. *Science* **308,** 1164–1167.
6. Irizarry, R. A., Warren, D., Spencer, F., et al. (2005) Multiple-laboratory comparison of microarray platforms. *Nat. Methods* **2,** 345–350.
7. Larkin, J. E., Frank, B. C., Gavras, H., Sultana, R., and Quackenbush, J. (2005) Independence and reproducibility across microarray platforms. *Nat. Methods* **2,** 337–344.

8. Sambrook, J., Fritsch, E. F., and Maniatis, T. *Molecular Cloning, A Laboratory Manual,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

9. Kamath, R. S., Fraser, A. G., Dong, Y., et al. (2003) Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* **421,** 231–237.

10. Timmons, L., Court, D. L., and Fire A. (2001) Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. *Gene* **263,** 103–112.

11. Lewis, J. A. and Fleming, J. T. (1995) Basic culture methods. *Methods Cell Biol.* **48,** 3–29.

12. Lamitina, S. T., Morrison, R., Moeckel, G. W., and Strange, K. (2004) Adaptation of the nematode *Caenorhabditis elegans* to extreme osmotic stress. *Am. J. Physiol. Cell. Physiol.* **286,** C785–C791.

13. Mello, C. C. and Fire, A. (1995) DNA transformation. In: Caenorhabditis elegans*; Modern Biological Analysis of an Organism,* (Epstein, H. F. and Shakes, D. C., eds.), Academic Press, San Diego, CA, pp. 452–482.

14. Romagnolo, B., Jiang, M., Kiraly, M., et al. (2002) Downstream targets of let-60 Ras in *Caenorhabditis elegans*. *Dev. Biol.* **247,** 127–136.

15. Urano, F., Calfon, M., Yoneda,T., et al. (2002) A survival pathway for *Caenorhabditis elegans* with a blocked unfolded protein response. *J. Cell. Biol.* **158,** 639–646.

16. Nollen EA, Garcia SM, van Haaften G, et al. (2004) Genome-wide RNA interference screen identifies previously undescribed regulators of polyglutamine aggregation. *Proc. Natl. Acad. Sci. USA* **101,** 6403–6408.

17. Kelly, W. G. and Fire, A. (1998) Chromatin silencing and the maintenance of a functional germline in *Caenorhabditis elegans*. *Development* **125,** 2451–2456.

11

Assays for Toxicity Studies in *C. elegans* With Bt Crystal Proteins

Larry J. Bischof, Danielle L. Huffman, and Raffi V. Aroian

Summary

Caenorhabditis elegans is well suited for toxicological studies owing to its established biology, short generation time, large brood size, and readily scorable life traits. Quantitative parameters of *C. elegans* that can be assayed include growth, size, progeny production, behavior, and mortality. Qualitative parameters of toxicity, such as changes in appearance or movement, can also be determined. This chapter describes four assays we have used for analyzing the toxic effects of *Bacillus thuringiensis* crystal proteins toward *C. elegans*. The assays are quantitative growth measurement, brood size measurement, and determination of lethal concentration, as well as a qualitative health assay based on worm appearance. Although these assays are described for crystal proteins, the approaches are suited for the studies of other toxins with *C. elegans.*

Key Words: Toxicity assays; toxins; *Caenorhabditis elegans*; *Bacillus thuringiensis*; crystal proteins; growth assay; brood size; LC_{50}.

1. Introduction

The ease of culturing the nematode *Caenorhabditis elegans*, its well-studied biology, and its attributes of short development time and large brood size make this an excellent organism for toxicological and pathogenic studies. These attributes allow for large numbers of animals to be studied in a relatively inexpensive and rapid manner. *C. elegans* has also proven itself a model for biological studies relevant to higher animals in such areas as genetics, cell death, neuroscience, aging, and development *(1–4)*. Thus, toxicological results in *C. elegans* are likely relevant to higher animals. In addition, the ability to perform both forward and reverse genetic screens in *C. elegans (5,6)* allows for molecular insight into both the mechanism of action of a toxin and how *C. elegans* may respond to protect itself from the toxin.

Growth, development, reproduction, life span, and behavior of this animal are all well studied and can be used as quantifiable parameters of its health. For example, toxicological studies of *C. elegans* using metal and organic compounds have analyzed toxicity by lethal dose, brood size, body length, and movement *(7–10)*. *C. elegans* has also been used extensively to analyze the effects and modes of action for a variety of pharmacological agents *(11)*. As one example, the antiparasitic drug ivermectin was compared in wild-type and resistant *C. elegans* strains by use of a development inhibition assay as a measure of toxicity *(12)*.

In addition to pharmacological agents, *C. elegans* has been used in the study of interactions between the nematode and bacteria and fungi *(13–15)*, which the worm consumes. In some instances, there is a clear infection process by the microorganism, whereas in other cases the pathogenicity can be attributed to a specific toxin *(16)*. A common assay for killing by an intact bacterium is monitoring the time to death of *C. elegans* while feeding on the bacterium *(17)*.

The ability to study interaction of a specific bacterial toxin with *C. elegans* is provided by the Gram-positive bacterium *Bacillus thuringiensis* (Bt), a likely natural pathogen of *C. elegans* *(18,19)*. Bt is a ubiquitous soil bacterium that can target and kill nematodes using one dominant class of virulence factors, pore-forming toxins named crystal (Cry) proteins. During sporulation each Bt strain produces a few Cry proteins at high levels that aggregate to form crystals. More than 200 Cry protein variants are known. Individual Cry proteins have long been known to be toxic toward various insects, and a few Cry proteins are currently being used in transgenic crops for insect control *(20,21)*. More recently, four Cry proteins were found to intoxicate nematodes, including *C. elegans* *(19,22)*. Although assays for the measurement of toxicity of a specific nematicidal Cry protein, Cry5B, toward *C. elegans* is the subject of this chapter, the methods presented are generally applicable to the study of other toxins using *C. elegans*.

The use of genetics in *C. elegans* can facilitate the understanding of both how Cry5B can lead to intoxication as well as how the worm may have evolved defenses to protect against this pore-forming toxin. For example, the isolation and characterization of *C. elegans bre* (<u>B</u>acillus-toxin <u>re</u>sistant) mutants that are resistant to intoxication by Cry5B led to the identification of glycolipids as receptors for this toxin *(22–25)*. To gain insight into the worm's response to ingestion of Cry5B, a microarray experiment was performed to monitor changes in gene regulation *(26)*. This led to the identification of the p38 mitogen-activated protein kinase pathway and two downstream genes as being involved in a protective response against the toxin. Worms with defects in this pathway have hypersensitive phenotypes in that they are severely intoxicated by low doses of Cry5B that do not obviously intoxicate wild-type worms.

In the process of both determining nematicidal Cry protein toxicity toward *C. elegans* and comparing toxicity among resistant, hypersensitive, and wild-type strains, four toxicity assays were developed and used *(19,22–24,26)*. These include quantitative growth, brood size, and lethal dose assays performed in liquid media and a qualitative health assay performed on solid medium ("plate" assay). Although all assays provide useful toxicological information, there are some differences among them that may allow one assay to be more suited for a specific application. The growth assay begins with worms at the first larval stage (L1) and incubates them in liquid conditions for approx 2.5 d that allows for them to grow to early adulthood in the absence of toxin. Size of the worms is determined and compared by taking photographs and calculating the area of the worm using an outlining program *(24,26)*. This assay is unique among the others in that the worms can potentially progress through all four larval stages and, therefore, measures parameters of development as well as growth. This could be advantageous because if the toxin were more efficacious at the L1, L2, or L3 larval stages, this assay would be the only one of the four able to detect this.

The second assay, brood size, is particularly relevant from an ecological standpoint as it measures the ability of a toxin to prevent production of the next generation of animals. Both the brood assay and growth assay are more sensitive than the lethal concentration assay with Cry5B as inhibitory effects on brood size or growth occur at lower concentrations of Cry5B than those required to kill worms *(19,22,26)*. A similar effect was seen with ethanol when comparing its toxicity on brood size and worm death *(10)*. Therefore, if toxin amounts are limiting, either the brood size or growth assays could be better candidates than the lethal dose assay. The brood size assay can be performed by either determining the complete brood size (total number of progeny) over a period of 5–6 d *(22)* or by determining a partial brood size after 3 d *(19,23)*. For a complete brood size, the original test worms must be moved to new wells after 3 d or the early progeny will begin having progeny of their own, which would interfere with the counts. Therefore, a 3-d count is technically more feasible as it does not involve moving the parental worm to another test well.

The lethal dose assay is a standard toxicological assay that allows for determining the concentration of Cry5B at which 50% of the animals are killed (LC_{50}). One advantage of this assay is that owing to the relative ease of scoring worms as dead or alive, a large sample size can be analyzed. An important parameter for this type of assay is the time point at which death is determined. For several of the LC_{50} assays performed with heavy metals, time points of 24–48 h were used *(8,27)*. However, as Cry5B intoxicated worms have a slower onset of death, the assay is performed for 5–6 d, which is typical of studies of Cry toxicity against insects as well. The short generation time of *C. elegans* (3 d at 20°C) raises the problem of the offspring from the tested worms develop-

ing, which would interfere with the scoring. This issue can be overcome with at least two approaches. First, a temperature-sensitive sterile mutant can be used and grown at the nonpermissive temperature so progeny are not produced. The *fer-1* worm (which is sterile at 25°C) was used successfully in an LC$_{50}$ assay with Cry5B *(22)*. A disadvantage of this approach for screening mutants for altered sensitivity to the toxin is that double mutants need to be generated with the temperature-sensitive sterile mutant. Therefore, a faster alternative is to use an inhibitor of DNA synthesis to prevent production of viable eggs. One compound previously used in *C. elegans* for this application is 5-fluorodeoxyuridine (FUdR *[28,29]*). However, as FUdR at sufficiently high doses can affect some physiological aspects of *C. elegans (30)*, FUdR should be tested for an interaction with the toxin being studied on the worm before proceeding with this approach.

Finally, the ability to express Cry5B in *Escherichia coli* allows assays to be performed on plates using toxin-expressing bacteria as a food source *(19,26)*. The relative dose of Cry5B is determined by mixing *E. coli* that do not express Cry5B with *E. coli* that do express Cry5B prior to spreading on a worm growth plate. For example, a 10% Cry5B plate is one that has nine parts non-Cry5B expressing *E. coli* mixed with one part Cry5B expressing *E. coli*. The level of worm intoxication is determined by appearance, such as coloration, size, movement, and presence of fertilized eggs. Although the least quantitative of the assays, the plate assay is simple to perform, reproducible, allows for relative comparison of intoxication between strains, and can be used to rapidly screen worms by either forward (mutagenesis) or reverse (RNA interference) genetics.

2. Materials

General materials that are used in more than one assay are described first followed by materials that are specific for each of the assays. Also, more detailed instructions for *C. elegans* maintenance and growth are available *(31,32)*, but reagents sufficient for worm maintenance with respect to the toxicity assays are described.

2.1. Bacterial Strains and Reagents

1. *E. coli* OP50 (available from the *Caenorhabditis* Genetics Center at the University of Minnesota, http://biosci.umn.edu/CGC/CGChomepage.htm).
2. *E. coli* JM103 (American Type Culture Collection, Manassas, VA) containing an empty pQE9 vector (Qiagen Inc., Valencia, CA).
3. *E. coli* JM103 containing Cry5B inserted into pQE9 *(19)*.
4. Bt expressing Cry5B (for details of Cry5B purification *see* **ref. 23**).
5. Carbenicillin stock: 50 mg/mL in 50% EtOH, filter-sterilized.
6. Isopropyl β-D-1-thiogalactopyranoside (IPTG) stock (0.5 *M* in water, filter-sterilized).

2.2. Reagents for C. elegans

1. Nematode growth (NG) plates. To 972 mL of double distilled water (ddH$_2$O), add 3 g NaCl, 2.5 g bacto peptone (Becton, Dickinson, and Company, Sparks, MD) and 20 g bacto agar (Becton, Dickinson, and Company). Autoclave inside a flask containing a stir bar. After autoclaving, mix solution on a stir plate. When solution has cooled to about 50°C, add the following sterile reagents: 1 mL of 5 mg cholesterol/mL (prepared in ethanol), 1 mL 1 *M* CaCl$_2$, 1 mL 1 *M* MgSO$_4$, and 25 mL 1 *M* potassium phosphate (pH 6.0). Aliquot 12 mL of solution per 60-mm Petri dish. After plates have dried for 1–2 d, add a few drops of a saturated *E. coli* OP50 culture as a food source.

2. Enriched nematode growth (ENG) plates. Prepared as for NG plates except contain 5 g bacto peptone and 1 g bacto yeast extract (Becton, Dickinson, and Company) for 972 mL of water. 40 mL are added per 100-mm dish. Approximately 250 μL of an *E. coli* OP50 culture are spread per plate and incubated overnight at 37°C.

3. S basal: 0.1 *M* NaCl, 0.05 *M* KHPO$_4$ (pH 6.0). Prepare in ddH$_2$O, autoclave, and store at room temperature.

4. Trace metals solution: 5 m*M* EDTA, 2.5 m*M* FeSO$_4$, 1 m*M* MnCl$_2$, 1 m*M* ZnSO$_4$, 0.1 m*M* CuSO$_4$. Prepare in ddH$_2$O, autoclave and store in the dark at room temperature.

5. S medium: prepare by combining 10 mL S basal with 0.1 mL 1 *M* potassium citrate (pH 6.0), 0.1 mL trace metals, 0.03 mL 1 *M* CaCl$_2$, 0.03 mL 1 *M* MgSO$_4$, and 0.01 mL 5 mg cholesterol/mL (in ethanol). All components are sterile.

6. M9 buffer: 2.2 m*M* KH$_2$PO$_4$, 4.2 m*M* Na$_2$HPO$_4$, 85.6 m*M* NaCl, 1 m*M* MgSO$_4$. Prepare in ddH$_2$O without the MgSO$_4$ and autoclave. Then add in sterile MgSO$_4$ and store at room temperature.

7. Bleaching solution: prepare fresh by combining 3.5 mL ddH$_2$O, 0.5 mL 5 *M* KOH, and 1 mL 4–6% sodium hypochlorite.

2.3. L1 Growth Assay

1. 24-Well plate (Becton Dickinson Laboratories, Franklin Lakes, NJ, cat. no. 353047).

2. Chloramphenicol stock (10 mg/mL).

3. 2% Agarose solution in M9 buffer.

4. 10% Sodium azide stock in water (toxic; handle with care).

5. Microscope slides and cover slips.

6. Two microscope slides with a single layer of tape adhered to the top surface of each.

7. Compound microscope with ×10 objective lens and attached camera. The camera and software system must be able to capture an entire adult worm as a single image and be able to save the image as either a .jpeg or .tiff file.

8. National Institutes of Health (NIH) image: downloadable at http://rsb.info.nih.gov/nih-image/.

For Macintosh computers, download NIH Image 1.63. For Windows operating systems, download Image J 1.33.

2.4. Brood Size Assay

1. 96-Well plate (Becton Dickinson Laboratories, cat. no. 353072).
2. Artificial eyelashes.
3. Wooden pick (approx 15 cm long).

2.5. LC_{50} Assay

1. 48-Well plate (Becton Dickinson Laboratories, cat. no. 353078).
2. 5-Fluoro-2'-deoxy-uridine (Sigma, St. Louis, MO, cat. no. F0503). Dissolve in ddH$_2$O at 100 mM stock initially, dilute to 8 mM, and store at –20°C. FUdR is toxic and should be handled with care.
3. 0.1% Triton X-100.
4. Nine-depression glass spot plate (Corning Life Sciences, Acton, MA, cat no. 7220-85).

2.6. Cry5B Plate Assay

1. ENG-IC plates. Prepare ENG medium as in **Subheading 2.2.**, and when solution has cooled to approx 50°C, add carbenicillin to 50 μg/mL and IPTG to 0.1 mM. Add 12 mL of the medium per 60-mm dish. Store plates at 4°C.
2. Microscope capable of photographing worms.

3. Methods

An important consideration for all assays is that as mutants may grow or behave differently than wild-type worms, all worms should be normalized to the respective assay parameter in the absence of toxin. In addition, a wild-type worm control should always be performed at the same time when studying any mutant owing to inherent variability in the assays.

3.1. Bleaching C. elegans *and Preparing Worms for Assays*

1. Wash a 60-mm NG plate full of gravid adults twice with 2 mL H$_2$O. Combine the washes in a 15-mL conical tube and centrifuge at 500g for 45 s.
2. Aspirate supernatant, add 5 mL of H$_2$O, and spin as before. Again aspirate supernatant. Add 2 mL of bleaching solution and mix by hand. Monitor worm lysis on a dissecting scope and mix occasionally. When worms have fully lysed and eggs are released (typically takes 4–8 min), add 5 mL of H$_2$O. Centrifuge and aspirate as before. Wash with additional 5 mL H$_2$O. Then wash with 5 mL of M9. As the eggs do not pellet as well in M9 as water, increase spin to 75 s at 750g. Carefully pipet off supernatant and repeat 5 mL M9 wash. Following this last wash, resuspend eggs in 1 mL M9. Place tube on a rotary platform and allow eggs to hatch overnight at room temperature.
3. Count worms by pipetting several 5-μL aliquots onto a microscope slide. Examine on a dissecting microscope to determine the number of worms.
4. If worms will be used at the L4 stage, place L1 worms on ENG plates spread with *E. coli* OP50. Approximately 20,000 L1 worms can be placed on a 100-mm dish and grown until the L4 stage.

3.2. L1 Growth Assay

3.2.1. Setting Up Growth Assay

1. On day 1, inoculate 5 mL of Luria-Bertani (LB) medium with a single *E. coli* OP50 colony and incubate overnight at 37°C and 250 rpm. Also bleach gravid worms on d 1.
2. The assay is set up on day 2 using a 24-well plate. Each well will contain 40 μL of *E. coli* OP50 in S medium, 5 μL of L1 worms in M9 medium (30–40 worms total), 4 μL 100X toxin or control buffer, 1.2 μL chloramphenicol, and 350 μL S medium to bring total volume to 400 μL.
3. Specifically on day 2, pellet the *E. coli* OP50 culture at 2000g for 5 min, discard the supernatant and resuspend the pellet in an equal volume of S medium.
4. Spin L1 larvae for 45 s at 500g to gently pellet. Remove some of the supernatant leaving sufficient volume so there will be approx 6–8 worms/μL. Gently mix worms and count the number of worms in a 5-μL sample. If worms are too concentrated, add M9 until there are 30–40 worms per 5 μL. If worms are too diluted, re-spin and remove appropriate amount of supernatant.
5. Prepare the Cry5B protein to give a range of doses to obtain a complete response curve (from no effect on growth to maximal growth inhibition). For Cry5B, an initial dose range of 0.125 to 2.0 μg/mL is appropriate. Use serial dilution to prepare a 100X stock of each Cry5B concentration to be tested.
6. Assemble the wells, setting up three wells for each dose. Prepare a master cocktail (based on volumes for a single well described in **step 2**) containing S medium, *E. coli* OP50 in S medium, chloramphenicol and L1 worms. Add L1 worms just prior to aliquoting the cocktail into wells to prevent them from ingesting excessive *E. coli* OP50 prior to Cry5B exposure. Aliquot 396 μL of this master mix into each well. Add 4 μL of the 100X Cry5B solution or buffer control to each well. When addition of Cry5B is finished, timing of assay begins.
7. Wrap the plate in a damp paper towel and place inside an enclosed box at 20°C for 60 h. At this stage, N2 worms will be young adults (*see* **Note 1**).

3.2.2. Acquiring Worm Images

1. Prepare an agarose pad on a microscope slide. First melt the 2% agarose in M9 buffer in a microwave, then add sodium azide to 0.1% (use gloves as azide is toxic). Then, align a microscope slide between the two microscope slides with tape on their surface. The slides are parallel to one another, and the middle slide abuts the two slides with tape. Then place a drop of agarose on the middle slide (no tape) and quickly place another slide on top of the agarose drop. The top slide should be perpendicular to the orientation of the bottom slides so it is resting on the two slides with tape and the middle slide. Gently remove the top slide after about 1 min. The result will be a spot of agarose on the middle slide, and its thickness will correspond to the depth of the tape.
2. At the end of the growth period, use a glass Pasteur pipet to transfer the contents of the wells into 1.5-mL microfuge tubes. Pool the triplicate wells for each condition into a single tube.

Fig. 1. Worm outlining for growth assay. This is an example of a worm that has been photographed and then outlined using the National Institutes of Health Image program. The outlining is approximated as close as possible to the edge of the worm's body.

3. Gently pellet the worms by centrifuging the tubes with the tops open (prevents worm pellet from being disturbed after the centrifugation) by placing the microfuge tubes inside a 15-mL conical tube. Spin in a clinical centrifuge for 30 s at 450g.
4. Carefully pipet off supernatant leaving about 100 µL of solution.
5. Gently mix worms, pipet 3–5 µL onto a 2% agarose pad and add a cover slip (*see* **Note 2**).
6. Photograph at least 20 worms for each condition at ×100 magnification on a compound microscope. Save the images as either .jpeg or .tiff files.

3.2.3. Measuring Worms and Analyzing Data

1. Open each file in NIH Image for Macintosh (Image J for Windows). For each worm pictured in the file, outline the worm using the segmented line tool for Macintosh (polygon selection tool for Image J; **Fig. 1**). After the worm area has been selected with this tool, click on "Analyze" on the upper tool bar and select "Measure." In the "info" box for NIH Image (the "results" box for Image J), the area of the selection is given in number of square pixels. Record this value for each worm.
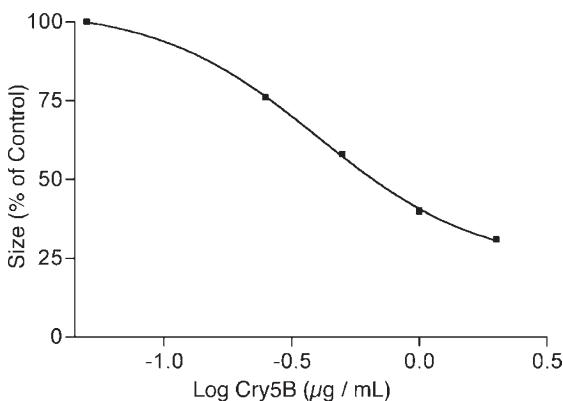
Fig. 2. Growth assay. The graph depicts a single L1 growth experiment using wild-type N2 worms with different concentrations of Cry5B. Size of the worms in the absence of toxin was set at 100%.

2. Calculate the average area in square pixels for each condition (*n* should be at least 20).
3. Normalize the average area at each toxin concentration to the average area of the no toxin control. This is done by dividing the average area at a specific Cry5B concentration by the average area at concentration zero. This yields the relative toxin effect and normalizes any natural differences in size between worm strains. Three trials of the assay should be performed to calculate a standard error (*see* **Fig. 2** for an example growth curve).

3.3. Brood Size Assay

This assay has been performed with both Cry5B crystal spore lysates from Bt cultures *(22)* as well as with Cry14A expressed inside *E. coli (23)*. However, in principle this assay should work with any purified Cry toxin or any soluble toxin.

1. Attach an artificial eyelash to a wooden pick by dipping the tip of eyelash in fingernail polish and then applying eyelash to end of pick. Trim eyelash so approx 3 mm extends from end of pick. Prepare a batch of such picks, wrap in aluminum foil and autoclave to sterilize (*see* **Note 3**).
2. On day 1, bleach gravid worms.
3. On day 2, plate L1 worms on an ENG OP50 plate and incubate at 20°C for 44 h so the worms will be at the L4 stage when assay is initiated (*see* **Note 4**).
4. On day 3, inoculate individual 5-mL LB solutions containing 50 µg carbenicillin/mL with single colonies of JM103 containing pQE9, and JM103 containing pQE9-Cry5B. Grow cultures 14–18 h at 37°C and 250 rpm.
5. On day 4, dilute 1 mL of overnight cultures into 7 mL LB, and add carbenicillin to 50 µg/mL. Incubate for 1 h at 37°C and 250 rpm.

6. Add IPTG to 50 μM final concentration and incubate culture for 3 h at 30°C and 250 rpm to induce protein expression.

7. Measure absorbance at 600 nM of both cultures and adjust them to the same absorbance using LB medium. Centrifuge 1 mL of each culture at 2000g for 5 min and resuspend in 1 mL of S medium.

8. Combine the pQE9 and pQE9-Cry5B cultures in different ratios to obtain a dose–response series of Cry5B. For example, perform twofold serial dilutions of the pQE9-Cry5B culture using the pQE9 culture as the diluent (*see* **Note 5** for estimation of Cry5B concentration). In this manner, the same amount of bacteria is always added to the well but the amount of Cry5B will vary depending on the dilution. Use pQE9 culture as the no toxin control.

9. The assay is set up on d 4 in 96-well plates in S medium with each well containing a single L4 worm and 10 μL of bacterial culture in a total volume of 120 μL.

10. To test 5 wells at each Cry5B condition, prepare a cocktail sufficient for 6 wells: combine 60 μL of bacterial culture in S medium, 2.2 μL chloramphenicol, and 657.8 μL additional S medium. Mix well and distribute 120 μL into 5 wells of a 96-well plate.

11. Individually pick L4 worms to each well. This is accomplished by placing a 2-μL drop of S medium onto a worm on a plate. Then the eyelash pick is used to gently go beneath the worm and lift it from the liquid. The worm is then transferred to the well and allowed to dislodge from the pick. The same pick can be used for each specific toxin condition.

12. When all worms have been transferred, place the multiwell plate in a plastic box containing a damp paper towel to provide humidity. Incubate at 25°C for 3 d.

13. After 3 d, count the number of progeny in each well. Use a Pasteur pipet to transfer the contents of each well as five or six drops onto an empty NG agar plate (without *E. coli)* and then count worms in the drops.

14. Repeat the entire assay a minimum of three times to calculate a dose–response of Cry5B on brood size.

3.4. LC$_{50}$ Assay

1. On day 1, bleach gravid populations of worms.

2. On day 2, add synchronized L1 worms to a 100-mm ENG OP50 plate and record number of worms. Grow N2 worms at 20°C for 44 h so they will be at the L4 stage on day 4 (*see* **Note 4**).

3. On day 3, inoculate 5 mL of LB medium with a single *E. coli* OP50 colony. Incubate 14–18 h at 37°C and 250 rpm.

4. Set up assay on d 4. Assays are set up in a 48-well plate format in triplicate for each Cry5B concentration and control. A single well consists of approx 20 L4 worms in 200 μL S medium containing OP50 at a final absorbance 600 nm of 0.6, 200 μM FUdR (*see* **Note 6**) and a specific Cry5B concentration.

5. After 44 h, collect worms by washing plate with 5 mL water and transfer worms to a conical tube with a sterile glass Pasteur pipet. Wash plate with an additional 5 mL of water and combine with first wash. Pellet worms at 500g for 45 s.

Aspirate supernatant and wash worms with 5 mL of S medium as with prior spin.

6. Resuspend worms in a volume of S medium to give approximately four worms per microliter based on starting number of worms added per plate. Count worms and adjust volume as necessary to give four worms per microliter.

7. Measure absorbance at 600 nm of overnight OP50 culture. Pellet OP50 at 2000g for 5 min. Resuspend bacteria in S medium to give absorbance at 600 nm of 3.0 ± 0.1.

8. Prepare a dose-range of Cry5B at 20-fold higher concentrations than will be tested in the wells. A typical starting range of Cry5B to test is from 0.625 to 40 µg/mL. Serially dilute the highest Cry5B concentration in twofold increments to achieve the dose-range.

9. A single well consists of 140 µL S medium, 40 µL OP50 in S medium at absorbance 600 nm = 3.0, 5 µL 8 mM FUdR, 10 µL of either 20 mM HEPES (pH 8.0) or desired Cry5B dose (in 20 mM HEPES buffer) and 5 µL of L4 worms. To set up each condition in triplicate, prepare a 3.5X cocktail of all components except for worms. Mix well and aliquot 195 µL into each of 3-wells on the 48-well plate. Just prior to adding the worms, add 1/100 vol of 0.1% Triton X-100 to the worms to give final concentration of 0.001% Triton-X100 (*see* **Note 7**). Then pipet in 5 µL of worms per well and pipet gently up and down twice to rinse any worms stuck to tip.

10. Place tray in an enclosed box that contains a moist paper towel to provide humidity. Incubate worms at 25°C for 5 d.

11. Determine worm viability based on movement. Transfer contents of each well with a glass Pasteur pipet to a well of a spot plate. A visibly moving worm is marked as alive. Worms that are not moving are gently touched with a platinum pick and watched for movement. Worms that fail to respond after several touches are marked as dead.

12. Data is plotted as percent of worms alive vs Cry5B concentration to determine the LC$_{50}$ concentration. The entire assay should be repeated a minimum of three times for each strain (*see* **Fig. 3** for an example graph).

3.5. E. coli *Expressed Cry5B Plate Assay*

1. Inoculate individual 5 mL LB solutions containing 50 µg carbenicillin/mL with single colonies of JM103 containing pQE9 and JM103 containing pQE9-Cry5B. Grow cultures 14–18 h at 37°C and 250 rpm.

2. Dilute 0.5 mL of overnight culture into 4.5 mL LB, and add carbenicillin to 50 µg/mL. Incubate for 1 h at 37°C and 250 rpm.

3. Add IPTG to 50 µM final concentration and incubate culture for 3 h at 30°C and 250 rpm to induce protein expression (*see* **Note 8**).

4. Measure absorbance at 600 nm of each culture. Add LB medium to adjust each culture to absorbance at 600 nm equal to 2.0 +/– 0.1.

5. For a no toxin control plate, add 30 µL of JM103 containing pQE9 per 60 mm ENG-IC plate and spread using sterile technique. Do not spread to edges of plate
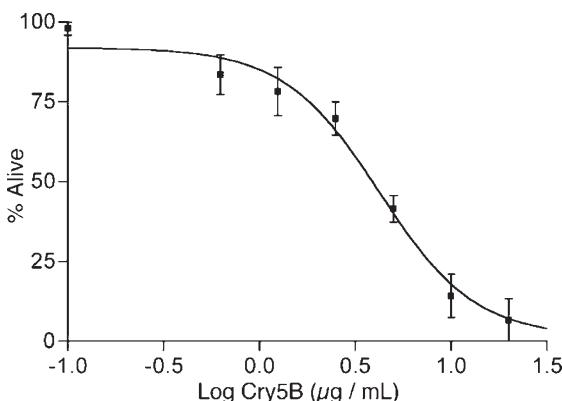
Fig. 3. Lethal concentration assay. This graph depicts a single LC_{50} assay in which the number of worms alive after 5 d of exposure to a dose series of Cry5B was determined. The graph represents a single experiment with each Cry5B concentration tested in triplicate to produce standard deviations.

to help prevent worms from leaving the plate. For a 100% Cry5B expressing lawn, add 30 µL of JM103 containing pQE9-Cry5B per 60-mm ENG-IC plate and spread using sterile technique. To obtain diluted Cry5B expressing lawns, add Cry5B expressing *E. coli* to the pQE9 containing *E. coli*. For example, to obtain a 10% Cry5B plate, mix 900 µL of JM103 containing pQE9 with 100 µL JM103 containing pQE9-Cry5B. Spread 30 µL per 60 mm ENG-IC plate.

6. Incubate plates overnight at 25°C. Plates can be used for up to 5 d when stored at room temperature.
7. For worm assay, add 10–20 synchronized L4 worms per plate and incubate at 20°C.
8. Compare appearance of worms at 24–96 h to determine level of intoxication. Intoxicated worms will appear smaller, paler, less active, and have reduced pumping rates and fertilized eggs compared with healthy worms. Photograph worms to obtain images for records. *See* **Fig. 4** for comparison of resistant, hypersensitive and wild-type worms on toxin plates.

4. Notes

1. The aim of worm growth in the absence of toxin is to proceed from the L1 stage until young adulthood. For N2 worms, this is accomplished at 20°C for 60 h. A few eggs may be laid at this point but none will have hatched. As other strains may have different growth rates, the end point of young adulthood, again in the absence of toxin, should be the reference for 100% growth. Therefore, the time to reach this stage should be determined empirically for each strain.
2. The volume of liquid added will depend on the size of the agarose pad. Do not add too much liquid or the cover slip will push liquid and worms off the pad.
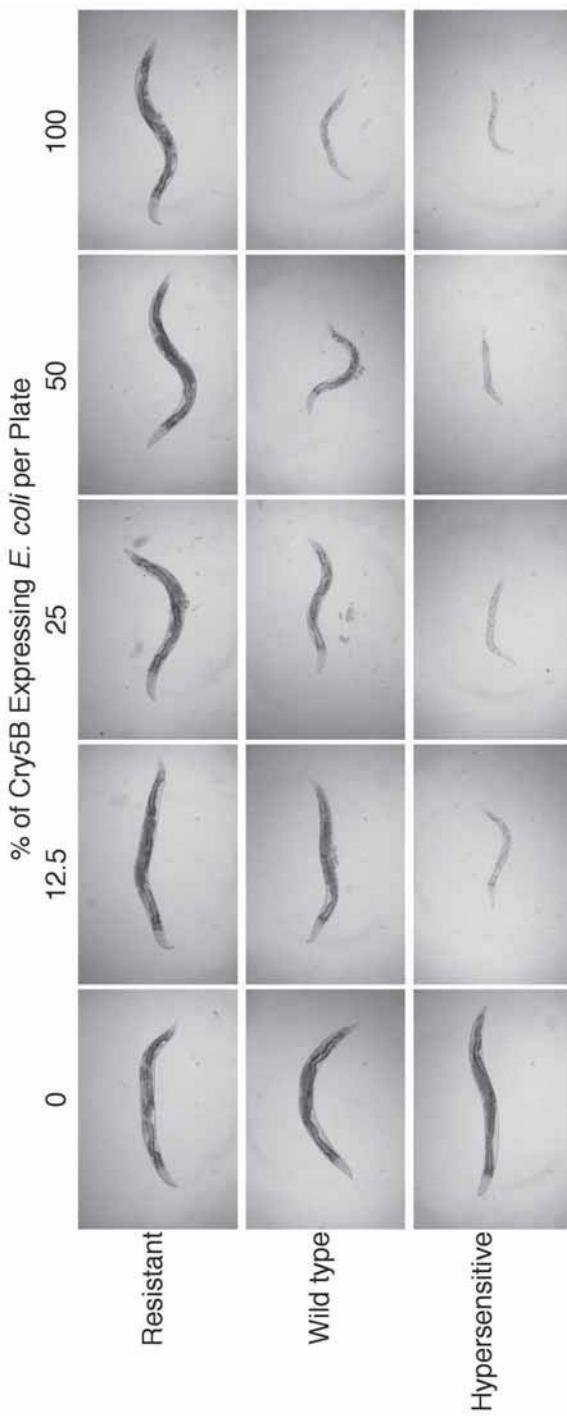
Fig. 4. L4 plate assay. Three strains of *Caenorhabditi elegans* were placed at the L4 stage on plates containing five different percentages of *Escherichia coli* that express Cry5B. Representative worms were photographed at ×100 magnification on 2% agarose pads 46 h after feeding on the bacterial lawns. The Cry5B resistant worms are large, dark, and healthy adults on all Cry5B plates. The wild type strain is large, dark, and healthy in the absence of toxin. In the presence of increasing Cry5B, the wild-type strain becomes more intoxicated as exhibited by it becoming progressively smaller and paler. The hypersensitive strain is healthy in the absence of Cry5B, but is severely intoxicated on all Cry5B expressing plates as noted by its small size and pale color. Although the wild-type worm is dark and large on the 12.5% Cry5B plate, the hypersensitive worm is small, pale, and clearly intoxicated.

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3. Movement of worms with the eyelash pick is gentler than a platinum pick and helps to avoid damage to the worm.
4. Growth of wild-type N2 L1 worms for 44 h at 20°C should yield worms at the L4 stage. Some mutants may grow at different rates, so when setting up these assays, determine the time required for the mutant to reach the L4 stage. If necessary plate the L1s of the different strains at appropriate times so they will reach the L4 stage in a synchronized manner to facilitate setting up the assay.
5. The approximate amount of Cry5B in the induced cultures can determined by lysing 10–20 µL of the culture in sodium dodecyl sulfate gel loading buffer by boiling for 5 min. This lysate is analyzed on an 8% denaturing polyacrylamide gel with Coomassie blue staining against a concentration range of bovine serum albumin to compare the relative intensity of the Cry5B band.
6. The minimum concentration of FUdR should be used so no larvae hatch during the assay. It is recommended this be determined empirically with each toxin to be tested.
7. Triton X-100 is used to decrease worms from sticking to the tips. The final Triton X-100 concentration in the assay is 0.000025% and has not been found to cause any ill effects on the worms.
8. The time of protein induction, concentration of IPTG used for induction of the cultures as well as in the ENG-IC plates will need to be determined empirically for each protein to be expressed.

Acknowledgments

References

1. Brenner, S. (2003) Nature's gift to science (Nobel lecture). *Chembiochem.* **4,** 683–687.
2. Horvitz, H. R. (2003) Worms, life, and death (Nobel lecture). *Chembiochem.* **4,** 697–711.
3. Sulston, J. E. (2003) *Caenorhabditis elegans:* the cell lineage and beyond (Nobel lecture). *Chembiochem.* **4,** 688–696.
4. Kenyon, C. (2005) The plasticity of aging: insights from long-lived mutants. *Cell* **120,** 449–460.
5. Brenner, S. (1974) The genetics of *Caenorhabditis elegans. Genetics.* **77,** 71–94.
6. Jorgensen, E. M. and Mango, S. E. (2002) The art and design of genetic screens: *Caenorhabditis elegans. Nat. Rev. Genet.* **3,** 356–369.
7. Popham, J. D. and Webster, J. M. (1979) Cadmium toxicity in the free-living nematode, *Caenorhabditis elegans. Environ. Res.* **20,** 183–191.

8. Williams, P. L. and Dusenbery, D. B. (1988) Using the nematode *Caenorhabditis elegans* to predict mammalian acute lethality to metallic salts. *Toxicol. Ind. Health*. **4,** 469–478.

9. Traunspurger, W., Haitzer, M., Hoss, S., Beier, S., Ahlf, W., and Steinberg, C. (1997) Ecotoxicological assessment of aquatic sediments with *Caenorhabditis elegans* (Nematoda): a method for testing liquid medium and whole-sediment samples. *Environ. Toxicol. Chem*. **16,** 245–250.

10. Dhawan, R., Dusenbery, D. B., and Williams, P. L. (1999) Comparison of lethality, reproduction, and behavior as toxicological endpoints in the nematode *Caenorhabditis elegans*. *J. Toxicol. Environ. Health, Part A*. **58,** 451–462.

11. Rand, J. B. and Johnson, C. D. (1995) Genetic pharmacology: interactions between drugs and gene products in *Caenorhabditis elegans*. In: Caenorhabditis elegans*: Modern Biological Analysis of an Organism, Vol. 48.,* (Epstein, H. F. and Shakes, D. C., eds.), Academic Press, San Diego, CA, pp. 187–204.

12. Dent, J. A., Smith, M. M., Vassilatis, D. K., and Avery, L. (2000) The genetics of ivermectin resistance in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* **97,** 2674–2679.

13. Huffman, D. L., Bischof, L. J., Griffitts, J. S., and Aroian, R. V. (2004) Pore worms: using *Caenorhabditis elegans* to study how bacterial toxins interact with their target host. *Int. J. Med. Microbiol*. **293,** 599–607.

14. Schulenburg, H., Kurz, C. L., and Ewbank, J. J. (2004) Evolution of the innate immune system: the worm perspective. *Immunol. Rev*. **198,** 36–58.

15. Kim, D. H. and Ausubel, F. M. (2005) Evolutionary perspectives on innate immunity from the study of *Caenorhabditis elegans*. *Curr. Opin. Immunol*. **17,** 4–10.

16. Nicholas, H. R. and Hodgkin, J. (2004) Responses to infection and possible recognition strategies in the innate immune system of *Caenorhabditis elegans*. *Mol. Immunol*. **41,** 479–493.

17. Tan, M. W., Rahme, L. G., Sternberg, J. A., Tompkins, R. G., and Ausubel, F. M. (1999) *Pseudomonas aeruginosa* killing of *Caenorhabditis elegans* used to identify *P. aeruginosa* virulence factors. *Proc. Natl. Acad. Sci. USA* **96,** 2408–2413.

18. Schulenburg, H. and Muller, S. (2004) Natural variation in the response of *Caenorhabditis elegans* towards *Bacillus thuringiensis*. *Parasitology*. **128,** 433–443.

19. Wei, J. Z., Hale, K., Carta, L., et al. (2003) *Bacillus thuringiensis* crystal proteins that target nematodes. *Proc. Natl. Acad. Sci. USA* **100,** 2760–2765.

20. Schnepf, E., Crickmore, N., Van Rie, J., et al. (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev*. **62,** 775–806.

21. Whalon, M. E. and Wingerd, B. A. (2003) Bt: mode of action and use. *Arch. Insect Biochem. Physiol*. **54,** 200–211.

22. Marroquin, L. D., Elyassnia, D., Griffitts, J. S., Feitelson, J. S., and Aroian, R. V. (2000) *Bacillus thuringiensis* (Bt) toxin susceptibility and isolation of resistance mutants in the nematode *Caenorhabditis elegans*. *Genetics* **155,** 1693–1699.

23. Griffitts, J. S., Whitacre, J. L., Stevens, D. E., and Aroian, R. V. (2001) Bt toxin resistance from loss of a putative carbohydrate-modifying enzyme. *Science* **293,** 860–864.

24. Griffitts, J. S., Huffman, D. L., Whitacre, J. L., et al. (2003) Resistance to a bacterial toxin is mediated by removal of a conserved glycosylation pathway required for toxin-host interactions. *J. Biol. Chem.* **278,** 45,594–45,602.

25. Griffitts, J. S., Haslam, S. M., Yang, T., et al. (2005) Glycolipids as receptors for *Bacillus thuringiensis* crystal toxin. *Science* **307,** 922–925.

26. Huffman, D. L., Abrami, L., Sasik, R., Corbeil, J., Van Der Goot, F. G., and Aroian, R. V. (2004) Mitogen-activated protein kinase pathways defend against bacterial pore-forming toxins. *Proc. Natl. Acad. Sci. USA* **101,** 10,995–11,000.

27. Tatara, C. P., Newman, M. C., McCloskey, J. T., and Williams, P. L. (1998) Use of ion characteristics to predict relative toxicity of mono-, di- and trivalent metal ions: *Caenorhabditis elegans* LC$_{50}$. *Aquat. Toxicol.* **42,** 255–269.

28. Hosono, R. (1978) Sterilization and growth inhibition of *Caenorhabditis elegans* by 5-fluorodeoxyuridine. *Exp. Gerontol.* **13,** 369–374.

29. Gandhi, S., Santelli, J., Mitchell, D. H., Stiles, J. W., and Sanadi, D. R. (1980) A simple method for maintaining large, aging populations of *Caenorhabditis elegans*. *Mech. Ageing Dev.* **12,** 137–150.

30. Mitchell, D. H., Stiles, J. W., Santelli, J., and Sanadi, D. R. (1979) Synchronous growth and aging of *Caenorhabditis elegans* in the presence of fluorodeoxyuridine. *J. Gerontol.* **34,** 28–36.

31. Sulston, J., and Hodgkin, J. (1988) Methods. In: *The Nematode* Caenorhabditis elegans, (Wood, W. B., ed.), Cold Spring Harbor Laboratory Press, Plainview, NY, pp. 587–606.

32. Stiernagle, T. (1999) Maintenance of *C. elegans*. In: C. elegans*: A Practical Approach,* (Hope, I. A., ed.), Oxford University Press, Oxford, UK, pp. 51–67.

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Fluorescent Reporter Methods

Harald Hutter

Summary

The identification and cloning of the green fluorescent protein (GFP) from jellyfish marks the beginning of a new era of fluorescent reporters. In *Caenorhabditis elegans*, genetically encoded markers like the fluorescent proteins of the GFP family became the reporter of choice for gene expression studies and protein localization. The small size and transparency of the worm allows the visualization of in vivo dynamics, which increases the number of potential applications for fluorescent reporters tremendously. In combination with subcellular tags, GFP can be used to label subcellular structures like synapses allowing novel approaches to study developmental processes like synapse formation. Other fluorescent labels like small organic dyes, which are in widespread use in cell culture systems, are rarely used in *C. elegans* owing to difficulties in applying these labels through the impenetrable cuticle or eggshell of the animal. A notable exception is the use of lipophilic dyes, which are taken up by certain sensory neurons in the intact animal and can be introduced into the embryo after puncturing of the egg shell. This chapter covers the use of fluorescent dyes and fluorescent proteins in *C. elegans*. Emphasis is placed on microscopic techniques including wide field and confocal microscopy as well as time-lapse recordings. The use of fluorescent proteins as transgenic markers and image processing of fluorescence images are briefly discussed.

Key Words: GFP; CFP; YFP; DsRed; DiI; confocal microscopy; *C. elegans*; time lapse; fluorescent reporter; embryo.

1. Introduction
1.1. Intrinsically Fluorescent Proteins as Reporters for Gene Expression and Protein Localization

Caenorhabditis elegans was the first multicellular organism, where the green fluorescent protein (GFP) was used in transgenic animals to label cellular and

subcellular structures in vivo *(1)*. Soon after its discovery, GFP was engineered for improved brightness and different spectral properties (color variants) by selection of variants after random mutagenesis *(2–4)*. Although the blue variant (blue fluorescent protein) never came into widespread use because of its poor fluorescence yield combined with fast bleaching, the cyan variant (cyan fluorescent protein [CFP]), together with GFP gave a first opportunity to combine two color fluorescent proteins for double labeling experiments. Further attempts to generate a red fluorescent derivative of the original GFP failed. The variant with the "longest wavelength" excitation in use today is a yellow variant (yellow fluorescent protein [YFP]). Soon after their generation GFP variants were adapted by the Fire lab for the use as reporters in *C. elegans*. Over the years, the Fire lab has provided a large variety of GFP vectors for various purposes to the entire *C. elegans* community. To further expand the set of reporters additional fluorescent proteins were isolated from different species of corals *(5)*. This resulted in the identification of several red fluorescent proteins, distantly related to GFP, one of which gained widespread use (drFP583/DsRed). As with the original GFP, improved versions of the protein with higher fluorescence yield and less tendency to oligomerize were generated and are now commercially available. Owing to the spectral overlap it is difficult to use all color variants simultaneously, the most suitable combination being a triple label consisting of CFP, YFP, and DsRed.

Intrinsically, fluorescent proteins are used in *C. elegans* mainly for two purposes. First, they are used in combination with promoter elements to characterize the expression pattern of a gene of interest and second, as tags to visualize the in vivo dynamics of expression and localization of a protein of interest. The major advantage of intrinsically fluorescent proteins compared with previously used reporters like *lacZ* comes from the fact that no additional factors or procedures are required to visualize the reporter. Consequently the reporter can be used in vivo with living animals, allowing the direct observation of temporal dynamics in an anatomical environment undisturbed by fixation procedures. Consequently, GFP replaced *lacZ* as reporter of choice almost instantly. As more and more promoters were characterized GFP also became the marker of choice to visualize particular anatomical features like subsets of neurons or even subcellular structures like synapses. This allowed new types of genetic screens for certain developmental defects like synapse formation, which were impossible or at least much more tedious before. In combination with time-lapse recordings GFP labels are used to visualize the dynamics of organelles and subcellular structures (centrioles, chromosomes) during cell division. However, we only have started to exploit the full potential of GFP to study dynamic cell biological processes and the use of fluorescent proteins as reporters in cell and developmental biology is expected to increase even further in the future.

1.2. Small Fluorescent Organic Dyes
as Labels for Anatomical Structures

Besides intrinsically fluorescent proteins small organic dyes are used to label anatomical structures in *C. elegans*. A major problem for the delivery of these probes is the fact that *C. elegans* at all stages is surrounded by a cuticle (or egg-shell in case of the embryo), which is impenetrable for many substances. Structures accessible for fluorescent dyes in the intact animal are the digestive tract and sensory neurons with endings open to the environment. These can be readily labeled by bathing worms in solutions containing fluorescent dyes. The eggshell of embryos can be punctured with a laser allowing access of externally added substances. This has been used to label cell membranes in order to follow cell divisions and changes in the shape and arrangement of cells in the embryo during cell migration and morphogenetic movements. For most other purposes, the flexibility of genetically encoded tags like GFP for the controlled and stable delivery of the label outweighs the ease of staining procedures in *C. elegans* and has made intrincisally fluorescent proteins like GFP the label of choice in *C. elegans.*

2. Materials

2.1. Vectors Containing cDNAs for Fluorescent Proteins

The Fire lab vector kits are the major source for GFP vectors for use in *C. elegans* (www.ciwemb.edu/pages/resources.html). The kits are now distributed by Addgene (http://www.addgene.org/Andrew_Fire). The vector kits contain vectors with cDNAs of all relevant GFP variants. The following variants are recommended: GFP in this text refers to the S65C variant (named gf3 in the 1999 Fire kit). CFP corresponds to the Y66W N146I M153T V163A variant (gf15) and YFP is the S65G V68A S72A T203Y variant (gf42). Furthermore the vector kits contain variants with tags for subcellular localization (nuclear, mitochondrial), cassettes for GFP insertion into exons or introns as well as several promoters for expression in specific tissues. A recent development is the adaptation of reconstituted GFP (recGFP) to *C. elegans* (*6*). Fluorescent GFP protein can reassemble when N- and C-termini are expressed independently and recombined in vitro (*7*). Leucine zippers have to be attached to the N- and C-terminus to provide an interface for reassembly. The recGFP system can be used to generate more specific expression or localization tags. Venus-GFP, a variant with spectral properties similar to YFP but with faster maturation, is described by Nagai et al. (*8*). cDNAs for fluorescent proteins are also available through several companies. GFP variants as well as DsRed variants are available through Clontech (www.clonetech.com). Novel fluorescent proteins with spectral properties similar to the original GFP are available from several companies including Clontech, Stratagene (www.stratagene.com), and Evrogen (www.evrogen.com).

Table 1
Filter Sets for Imaging Fluorescent Proteins [a]

Fluorophore	Excitation filter	Beam splitter	Emission filter	Comment
CFP	436/20	455	480/40	For maximal single signal detection
CFP	436/20	455	470/30	For minimal crosstalk with GFP
GFP	470/40	495	500LP 525/50	For optimal single signal detection, use also for DiO
GFP	480/20	495	515/30 510/20	For minimal crosstalk with CFP
YFP	500/20	515	535/30	For minimal crosstalk
DsRed	565/30	585	620/60	For minimal crosstalk with YFP; use also for DiI

[a] Specification are given as central wavelength in nanometers and width in nanometers. We use Chroma HQ filter sets with the specifications indicated in the Table.

CFP, cyan fluorescent protein; GFP, green fluorescent protein; YFP, yellow fluorescent protein; DiO, lipophilic dye (D-275, Molecular Probes).

Vectors provided by these companies are typically tailored for use in mammalian systems and, therefore, not suitable for direct use in *C. elegans*.

2.2. Equipment and Reagents

1. Stereo microscope: the first step in the use of genetically encoded fluorescent markers like GFP is the generation of transgenic animals (not covered here) and their identification. For the second part (identification), a stereo microscope equipped for epifluorescene is required. Major stereo microscope manufacturers like Zeiss or Leica provide such equipment and older stereo microscopes can be upgraded with a system provided by Kramer Scientific Corporation (Valley Cottage, NY).

2. Wide field microscope: all major microscope manufacturers (Zeiss, Leica, Nikon, Olympus) produce equipment suitable for imaging fluorescent proteins. A key part, especially for clean separation of GFP variants, is the optical filters defining excitation and emission windows. *See* **Table 1** for details of recommended filter sets. A large selection of filters is available from Chroma Technology Corp, *see* Website: www.chroma.com for technical specifications and recommended use. For multifocal plane recordings and/or time-lapse recordings certain components of the microscope have to be motorized (stage, filter changer, shutter). This is usually the case in newer microscopes. Older microscopes can be retrofitted with third party equipment (contact the microscope manufacturer).

3. Confocal microscope: all confocal microscopes are suitable for imaging fluorescent proteins. The Leica SP2 system with acusto-optical beam splitter is quite

flexible when several fluorophores are used in combination. It is devoid of optical filters allowing the emission windows to be selected freely. With sequential excitation these feature allow a clean separation even of fluorophores with significant spectral overlap like the different GFP variants. The Zeiss LSM 510 Meta system uses a different approach to deal with spectral overlap. Here, an array of photomultipliers is used to collect spectral information for each channel using software algorithms to correct for crosstalk between channels. With both systems a satisfactory separation of all GFP variants can be achieved under optimal circumstances (enough signal in all channels). Spinning disk confocal microscopes allow faster scan rates combined with less damage owing to reduced sample illumination and are therefore ideally suited for time-lapse recordings.

4. Laser system: a nitrogen-pulsed dye laser system for attachment to a fluorescence microscope is available through Photonic Instruments (Arlington Heights, IL). Such a laser can be used to puncture the egg shell to allow access of fluorescent dyes to the embryo.

5. Other equipment: heating block (temperature range $\leq 100°C$) with holes that can hold tubes with agar solution (*see* **Subheading 3.1.**, **step 1**).

6. Consumables: microscopy slides, cover slips (between 18×18 and 24×24 mm), Pasteur pipets, glass tubes holding approx 10 mL of liquid, Eppendorf tubes, capillaries for mouth pipets (in their simplest form consisting of a capillary holder connected to a flexible tube connected to a mouth piece), watchmaker glasses.

7. Chemicals: agar–agar (any brand will do, e.g., Difco), Vaseline (e.g., Neolab), levamisole, sodium azide; lipophilic dyes from Molecular Probes: DiO (green, D-275), DiI (red, D-282), DiD (dark red, D-307).

8. M9 buffer (*see* **ref. *9***) per liter: 3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 mL 1 *M* $MgSO_4$; autoclave.

9. Embryonic growth medium (*see* **ref. *10***): add to 9 mL *Drosophila* Schneider Medium (Gibco BRL): 1 mL of 5 mg/mL inulin (Sigma), 50 mg polyvinyl pyrrolidone powder (tissue culture grade; Sigma), 100 µL each of BME Vitamins (Gibco BRL), chemically defined lipid concentrate (Gibco BRL) and 100X concentrated penicillin–streptomycin (Gibco BRL), final volume 10.3 mL. Prior to use add 35% (volume/volume) bovine fetal calf serum (Gibco BRL). Osmolarity should be 307 mosM. Adjust tonicity by varying the amount of fetal calf serum.

3. Methods

3.1. Preparation of Agar Pads for Mounting Animals

C. elegans animals are under internal pressure and tend to explode when simply put on a slide and covered with a cover slip. A simple solution to this problem is to provide a cushion in the form of a drop of agar solution flattened between two slides.

1. Boil 5 g agar–agar (e.g., from Difco) in 100 mL deionized water until the agar is dissolved and the solution becomes clear. Distribute the solution into 10-mL glass tubes (3–5 mL into each tube). Seal the tubes with Parafilm and store at –20°C. Can be stored for several months.

2. Before mounting: set a heating block that can hold your tubes to 80°C and put one tube of agar solution into the block to melt the agar (briefly heat the tube after thawing with a Bunsen burner to speed up the melting process). Keep a Pasteur pipet in a second tube with deionized water in the heating block (will be used to pipet the agar solution; should warm up, so that the agar does not solidify inside the pipet).
3. To prepare an agar pad put a drop of agar solution on a slide using the Pasteur pipet and flatten the drop immediately with a second slide. To prevent premature drying of the pad, prepare agar pads shortly before mounting and do not remove the second slide until you are ready to mount the animals. Alternatively store the slides in a humid chamber until use.
4. When ready to mount animals remove the top slide from the agar pad and place animals within a drop of solution onto the pad. Having the two slides perpendicular to each other facilitates separation of the slides.

3.2. Mounting Individual Animals
or Late Stage Embryos for Microscopy

1. Prepare an agar pad (*see* **Subheading 3.1.**), remove the top slide and add 5 μL M9 solution (containing anesthetic if required, *see* **Subheading 3.4.**).
2. Use a worm pick to collect individual animals from a culture plate and transfer them into the M9 solution. Transfer as little bacteria as possible. Repeat until you have collected enough animals and finally, add a cover slip.

3.3. Transferring a Larger Number of Animals Onto Agar Pads

1. To collect a larger number of animals flood an entire culture plate with 1–2 mL M9 solution (containing anesthetic if required, *see* **Subheading 3.4.**). Transfer the worms in solution to an Eppendorf tube. Worms will stick to plastic and using plastic pipets and Eppendorf tubes results in some loss of animals. Use glass pipets and collect animals in a watchmaker glass to avoid any loss.
2. Let animals settle for a few minutes or spin them gently in a centrifuge ($3000g$).
3. Distribute them onto several agar pads (about 5 μL solution per pad). Add the cover slip.

3.4. Using Anesthetics

To prevent animals from moving you can supplement the M9 solution with 3 mM levamisole or 10 mM sodium azide (NaN_3). Levamisole acts at the neuromuscular junction resulting in hypercontracted animals displaying a lateral aspect. They might still occasionally twitch and spoil image acquisition with long exposure times. NaN_3 eventually will kill the worms (after a few hours with clear signs of cellular degeneration preceding death), but completely prevents them from moving within a few minutes. Furthermore, animals will eventually become straight and stiff (this will take some time; check before adding the cover slip) and can, therefore, adopt any orientation (ventral, lateral, dorsal).

3.5. Recovering Animals From Slides

Sometimes GFP reporters are used to evaluate phenotypes and, in such cases, you might want to recover animals from slides.

1. If you want to recover animals from slides use either no anesthetic or levamisole. Try to avoid having too many animals on the slide (animals should not lie in large groups and not on top of each other). Avoid too much liquid underneath the cover slip (some air should be left). Avoid too little liquid, as animals might be damaged by shear force upon removing the cover slip.
2. Locate the animal you want to recover under microscope. Go to the lowest magnification (×10 usually) and memorize the surroundings of the animal.
3. Take the slide to a stereo microscope (avoid moving the microscope stage when removing the slide from the microscope). Try to locate the desired animal under low magnification using the memorized landmarks. Be aware that certain microscopes might display a mirror image or might show an upside-down image of your slide. If you cannot find your animal, go back to the microscope. If you did not move the stage the desired animal should still be in the center of the field of view.
4. Once you have located your animal under the stereo microscope, slowly slide the cover slip to the side. If the animals start to slide as well you have too much liquid, if animals start to break up you do not have enough liquid.
5. Once the cover slip is removed transfer the worm to a culture plate using a worm pick. Animals dry out quickly under these conditions, so be quick. If you have used an anesthetic put a drop of M9 buffer (5–10 µL) onto the animal to wash out the anesthetic.

3.6. Puncturing the Eggshell With a Laser Beam to Allow Access of Fluorecent Dyes

The eggshell of *C. elegans* embryos forms a barrier preventing access of many substances like fluorescent dyes. The eggshell can be permeabilized by puncturing it with a laser beam allowing access of substances from the surrounding solution. A nitrogen-pulsed dye laser system for attachment to a fluorescence microscope is available through Photonic Instruments (Arlington Heights, IL).

1. Set up the laser attachment according to the manufacturers protocol. Center the laser and adjust the laser strength. The laser should be strong enough to crack the cover slip when focusing onto the cover slip.
2. Mount embryos on agar pads in embryo culture solution supplemented with the dye you want to use (e.g., the membrane dye FM 4-64 [Molecular Probes, Eugene, OR]).
3. Focus into the middle plane of the embyo onto a part of the eggshell that is "far" away from a cell membrane (usually the anterior end of the embryo). Fire a single shot at the eggshell. There should be a brief movement of cells toward the prospective hole if the eggshell was successfully punctured. There might be no obvious damage or hole. If unsuccessful, repeat the shot or increase laser strength. Cells shrinking or swelling after this operation or cells that stop dividing are an indication for a mismatch in osmotic strength of the solution surrounding the embryo.

4. The dye will diffuse through the hole in the eggshell. Embryos can be used for time-lapse recordings immediately afterward.

3.7. DiI Staining of Sensory Neurons

Some of the sensory neurons in *C. elegans* have endings that are open to the environment so that they can detect chemicals in the environment. If their anatomical structure is intact, they can be stained with lipophilic dyes, which are taken up from the surrounding liquid. Neurons staining under these conditions are ASK, ADL, AWB, ASH, ASI, ASJ, PHA, PHB. Lipophilic dyes come in various color variants (green, red, far red) that can be combined with other fluorescent markers like GFP variants.

1. Prepare a stock solution of the dye in a solvent according to manufacturers suggestions (typically dimethylsulfoxide or ethanol, e.g., 10 mg/mL for DiI). Before use spin the tube to precipitate undissolved particles.
2. Make a 1:1000 dilution of the dye in M9 buffer for staining worms.
3. Wash worms off from a culture plate with 1 mL M9 buffer, transfer them to an Eppendorf tube, and pellet them gently by centrifugation (3000*g*, 3–5 min).
4. Remove the supernatant and add 1 mL dye solution to the worm pellet. Leave worms in dye solution for approx 1 h in the dark.
5. Pellet worms, remove supernatant, and add 1 mL M9 buffer to wash out excess dye. Pellet worms again and repeat wash twice.
6. Transfer worms in as little liquid as possible to a new culture plate with food. Let worms crawl and feed for 3–5 h (the dye is also taken up by the gut, which stains intensely and will destain slowly when worms feed; check occasionally for gut staining).
7. Prepare agar pads and mount worms for fluorescence microscopy.

3.8. Wide-Field Fluorescence Microscopy

Imaging of fluorescent probes at a wide-field microscope (i.e., your standard fluorescent microscope) is straightforward with modern microscopes. A few recommendations for optimal image acquisition follow:

1. Choice of microscope: you do not need the latest, computerized model for viewing and documentation of still images. In the author's lab, people have the choice of a 12-yr-old Axioskop and a fancier, motorized Axioplan II (same manufacturer). Everybody wants to use the old Axioskop, because subjectively fluorescent images are significantly brighter than in the new microscope. Possible reasons are a longer light path in the newer microscope and a larger field of view leading to less light per area arriving at your eye piece. More important than the choice of microscope is the actual set-up, in particular the objective and filter sets used. Use objectives that are optimized for fluorescence microscopy (should be standard at your fluorescence microscope). Check that your lenses are clean, your fluorescence light source is properly aligned, the bulb is not too old, and your Nomarski looks good. If the Nomarski is bad, there might be a problem with the

light path that could have a general effect on the image quality even if you do not use Nomarksi. On Nomarski microscopes take out the upper polarization filter in fluorescence mode because you will not need it and it will significantly attenuate your fluorescence light. Get familiar with the details of your microscope, so that you are able to notice and correct adjustment problems.

2. Choice of filters: filter sets can be optimized either for optimal detection of a single fluorochrome or for optimal separation of fluorescence signals of different fluorophores. No single filter combination is ideal for both situations. Filters for visualization of a single marker like GFP alone have broad excitation and emission filter, allowing for a maximum of excitation light and a maximum of emitted light to pass the filters. By contrast filter sets designed to separate different fluorophores tend to have small excitation and small emission windows to prevent excitation of other fluorophores and to prevent fluorescent light from other fluorophores to pass the filters. Depending on spectral details of the fluorophores to be separated, these filters might even not be centered around the excitation and emission maxima, leading to further loss of signal with the advantage of better signal separation. Using narrow filters for the detection of a single fluorophore or using a wide filter in a combination of two markers can be a frustrating experience. Although filter sets are expensive, they are a one-time investment and not having the optimal filters can be an endless source of sorrow at the microscope.

3. Choice of imaging system: the core of an image documentation system at a modern microscope nowadays consists of a charge-coupled device (CCD) camera. Several manufacturers provide such cameras. For fluorescence microscopy cameras with technical specifications of a Hamamatsu Orca 100 or a PCO Sensicam are adequate to image nonmoving samples. If you are not sure whether the sensitivity of your camera is sufficient, take an image of an unlabelled pregastrulation stage embryo with a wide GFP filter set. The camera should be able to detect the background fluorescence giving you a picture of dimly fluorescent cells with a darker nucleus. CCD cameras have improved quite a bit over the last years. Major progress has been made to provide faster cameras with larger detectors (more pixels), progress in terms of increased sensitivity (quantum yield of detectors, signal-to-noise ratio) is less dramatic, so that even older CCD cameras are sufficient for standard documentation purposes. Digital detection systems for microscopy (cameras or photomultipliers) come with 8- or 12-bit dynamic range (corresponding to 256 or 4096 different gray values). Frequently sales representatives try to make the point that 8-bit dynamic range is sufficient and every time I demonstrate that for my samples (GFP labeled neurons with bright cell bodies and thin processes) this is not the case. In general I would aim for a 12-bit detection system, which also gives you more flexibility and is going to be the standard soon anyways. Cameras continue to evolve, so that specific recommendations are likely to be outdated soon. Different software packages exist to control image acquisition and data analysis. Software is developing rapidly as well, and features are comparable so that no specific recommendation can be made. Avoid proprietary image formats when storing images. You do not want to be stuck with a particular program to open and access your images.

3.9. Confocal Microscopy

In a confocal microscope fluorescence light coming from planes other than the current focal plane is blocked by a pinhole in the light path. This allows a better discrimination of fluorescence signals by removing the "haze" or "blur" typically seen in a wide-field microscope where light from all focal planes reaches the detector. In confocal microscopes a laser is used for fluorophore excitation, providing stronger excitation with all its advantages and disadvantages (more signal, more bleaching, and damage). Because of the pinhole samples have to be scanned and image aquisition is rather slow. Spinning disk confocal system use arrays of several thousand pinholes simultaneously, which significantly speeds up image acquisition (for image-lapse recording) but reduces signal strength (but also damage) because the laser light is split up to pass through all the pinholes simultaneously.

1. Choice of microscope: typically this is not an issue, because you will have to use whatever is around. Confocal microscopes have evolved significantly in the past decade, so here "newer" most certainly means more features and options and, therefore, probably "better." For single-channel imaging, confocal microscopes are generally well equipped. For multicolor imaging, different manufacturers follow different strategies (*see* **Subheading 2.**).

2. Data aquisition: fluorescent *C. elegans* samples are not different from any other fluorescent samples. Follow the recommendations of your imaging facility manager or confocal expert for the particular microscope you are using. One *C. elegans*-specific issue is autofluorecence, in particular of gut granula, which give a bright and often annoying background. In fact, they are one of the reasons for using a confocal microscope where they are already eliminated during image aquisition when they are in a different focal plane. Narrow GFP filters block most of the gut autofluorescence, which is strongest in the yellow part of the spectrum.

3.10. Time-Lapse Recordings of Embryos

Very few labs have used fluorescent reporters in *C. elegans* for time-lapse recordings. In all cases, embryonic development was studied. Microscope setups used often were custom modifications of commercial systems adapted to the particular experiment so that a general procedure cannot be extracted. However, there are some common aspects, which are outlined below. The major issue for time-lapse recordings is that prolonged illumination with excitation light damages the embryo. One solution is a reduction in the amount of light used for excitation. This is currently possible because the corresponding reduction in fluorescence can be compensated by more sensitive detection systems (typically cooled CCD cameras). The second solution is to use multiphoton excitation, which has the disadvantage of requiring equipment that is not ubiquitously available. The number of images that can be acquired before the embryo is damaged

and stops developing depends on the total exposure time (exposure per image times number of images taken).

3.10.1. Mounting Pregastrulation Stage Embyos for Time-Lapse Recordings

1. Fill a watchmaker glass with M9 buffer.
2. Transfer gravid adults into the glass with your worm pick.
3. Cut animals open with a scalpel, so that eggs will come out.
4. Prepare a slide with agar pad (*see* **Subheading 3.1.**), transfer embryos onto the agar pad with a mouth pipet in as little liquid as possible (<5 μL).
5. If you want to move eggs around, remove as much liquid as possible with the mouth pipet. Use an eyelash attached to a tooth pick to move eggs around on the agar pad.
6. Carefully put a cover slip (18×18) on top. Add M9 buffer to fill the space underneath the cover slip.
7. Seal the cover slip with Vaseline. Use a small paintbrush to distribute the Vaseline (you can keep one tube with molten Vaseline in the same heating block that holds your agar solution).

3.10.2. Recordings Using Wide-Field Microscopes

1. When using a mercury lamp for excitation attenuate the excitation light to about 5–10% of the maximum using neutral density filters. Short-term recordings with exposure times of 250 ms (using an Orca100 camera from Hamamatsu) and images acquired every 6–8 s for several minutes have been successfully done this way *(11)*.
2. Alternatively a halogen light source has been used for long term (up to hatching) multifocal plane recordings of GFP labeled embryos with a Zeiss Axioplan II microscope and a Hamamatsu Orca camera (Thomas Bürglin, personal communication, April 2005; Bürglin can be contacted at the Karolinska Institute for recording details http://www.biosci.ki.se/groups/tbu/).

3.10.3. Recordings Using Confocal Microscopes

1. On a spinning disk confocal microscope a 50-mW argon laser set to 40% of its maximum strength was used for short-term time-lapse recordings of early embryos *(11)*.
2. At a two-photon-setup a femtosecond-pulsed 1047 nm Nd:YLF laser on a modified MRC-600 Laser-Scanning Confocal System was used for multifocal plane time-lapse recordings of embryos stained with the membrane dye FM 4-64 and of GFP labeled embryos *(12,13)*.

4. Notes

1. Andy Fire observed that the introduction of artificial introns leads to increased expression levels of transgene reporters like GFP. Consequently all Fire vectors have these artificial introns within the coding region of GFP and also one intron each before the start and after the stop codon. Commercially available fluores-

cent proteins generally do not have these introns in their cDNAs. We find with DsRed and its derivatives that expression levels are comparable to GFP and its derivatives when the intronless cDNA is used to replace an intron containing GFP in a Fire vector. Apparently the two introns flanking the cDNA result in sufficient enhancement of expression.

2. Commercially available fluorescent proteins often have codon usages optimized for expression in mammalian cells. We find with GFP derivatives no significant effect on expression level (neither positive nor negative) of GFP variants optimized for mammalian codon usage.

3. Brightness of GFP variants: the different fluorescent proteins are not equally bright. Among the GFP variants CFP is significantly less bright than GFP or YFP, which are comparable in brightness. For single labels GFP or YFP, therefore, is preferable over CFP and for double labeling it may be advisable to combine the stronger expressing promoter with CFP to minimize detection problems.

4. DsRed variants: the original DsRed takes about 4 d to accumulate detectable fluorescence, which continues to increase further over the next few days. This makes DsRed a nice marker in older adult animals, but renders it more or less useless as marker for earlier stages in *C. elegans*. Its derivative DsRed2 reaches maximum brightness already in L1 stage larvae and is clearly detectable already in embryos. However, it still seems to take longer to mature than GFP variants, making it less ideal to detect the beginning of gene expression in the embryo. Subjectively DsRed2 is the brightest of all fluorescent proteins I have seen and could, therefore, be the marker of choice for single labels. However, when images are taken at the confocal microscope the comparatively weak green lasers and the reduced sensitivity of photomultipliers used for signal detection in the red part of the spectrum often makes image accquisition even of brightly red fluorescent probes a disappointing adventure. DsRed2 still has a tendency to aggregate and several labs reported that expressing large amounts of the DsRed has cytotoxic effects. Therefore, GFP or YFP are still the label of choice when only a single color is required.

5. Separation of CFP and GFP: CFP signals are detectable in the GFP filter sets (crosstalk) leading to problems distinguishing these two variants. However even strong CFP signals are virtually undetectable in the YFP filter, which still allows to detect much of the GFP signal, effectively leading to a clear separation of CFP and GFP.

6. Non-GFP filter sets: FITC filter sets are suitable for GFP/YFP visualization, Cy3/ TRITC/Rhodamine filter sets work for DsRed. They are an alternative when GFP filter sets are not available.

7. Multipass filter sets for simultaneous visualization of fluorescent probes: filter sets with multiple excitation and emission windows in theory allow simultaneous visualization of several fluorophores in different colors. In practice, this works well only for strong signals and only for combinations with comparable signal strength in all channels, i.e., for GFP/YFP in combination with DsRed, but not so

well with CFP combined with GFP/YFP and/or DsRed. The CFP signal is too weak and the substantial crosstalk between CFP and GFP make unequivocal identification of these variants difficult. A long pass GFP filter set can be used as multipass filter set for GFP and DsRed, i.e., the DsRed signal is quite visible in such a filter.

8. Detecting weak GFP expression: wide or even long pass emission filters are recommended when the question arises of whether there is any fluorescence signal at all. Gut autofluorescence in *C. elegans* appears yellow when viewed with a long pass filter and can be distinguished from the GFP signal by color. Color discrimination is not possible with a band pass filter set (everything is green then).

9. Soluble GFP: cells in *C. elegans* are small, so that soluble GFP readily fills the entire cell with all its processes (neurites, muscle arms). It is not necessary to add tags to GFP to obtain sufficient signal in thin processes far away from the site of synthesis. Soluble GFP seems to be tolerated even at high concentrations without an adverse effect on development or cell physiological processes. By contrast, targeted localization of GFP, for example, to cellular membranes can lead to problems, especially in combination with high expression levels. It is therefore advisable to avoid adding tags to GFP used simply to label subsets of cells. On the other hand, targeting of GFP to plasma membranes reduces signal levels in the soma, which are several orders of magnitude higher compared to thin cellular processes when soluble GFP is used. This is an advantage when processes close to cell somata have to be visualized.

10. Subcellular localization: when tags are used to target GFP to certain subcellular structures, expresssion levels of GFP have to be controlled. Strong overexpression frequently leads to saturation of the targeting machinery resulting in a substantial portion of the GFP not to be localized correctly. In extrachromosomal arrays of transgenic animals expression levels can be controlled indirectly by modulating the copy number of the transgene through addition of foreign non-expressing DNA. You might have to generate several lines to find a compromise with the optimal yet correctly localized signal.

11. Cell identification: shape and size of cell and the position of the cell body are features commonly used to identify cells in *C. elegans*. Because soluable GFP reporters fill the entire cell they are the reporter of choice for cell identification during characterization of novel expression patterns. Strong expression in large cells like pharyngeal muscle cells, however, can obscure expression in other cells like neurons that are close by. In such cases, targeting of the GFP to the cell surface or the nucleus can be advantageous. The shape of a cell often reveals a lot about the identity of the cell, so that soluble GFP rather than nuclear localized remains the first choice when cells have to be identified.

12. Commonly used markers to identify transgenic animals carrying reporter constructs are *rol-6(su1006)* (*14,15*), *pha-1(e2123ts)* (*15*), *unc-4(e120)* (*16*), or *lin-15(n765ts)* (*17,18*). In addition several labs have started to use GFP markers themselves as co-injection marker to allow easy identification of transgenic ani-

mals directly under the fluorescent microscope. The choice of co-injection marker obviously is influenced by nature of the experiments to be done with the transgenic strain and by the question of whether or not the transgene will be integrated at some point. If integration is the ultimate goal the use of a fluorecent co-injection marker has more disadvantages than advantages. After integration it is no longer required, but still is present and will give a fluorescent signal in one of the channels, restricting the subsequent use in combination with other fluorescent proteins. Because fluorescent co-injection markers are selected to be bright, crosstalk might become an issue and a serious problem when reporter expression is weak. With weak signals this might even be a problem for color combinations where you don't expect any (red as co-injection marker in combination with a green reporter). Most morphological markers like *rol-6* or *lin-15* cannot be recognized at all stages, in particular not in embryos, making it difficult to identify transgenic animals at certain stages. Furthermore (with the exception of *rol-6*) they are recessive and work only in combination with the mutated gene, making it more difficult to transfer them to other genetic backgrounds. On the other hand, fluorescent reporters as markers for transgenic animals are always dominant. *Pha-1* has the advantage of being a selectable marker (non-transgenic animals die as embryos), facilitating strain maintenance. For the characterization of unknown gene expression profiles this is the marker of choice in our lab. A ubiquitously expressed fluorescent marker, however, is helpful, when the transgenic strain is used for mosaic analysis, because every cell still carrying the transgene is labeled. When the transgene is integrated you might get away without any co-injection marker, provided your actual reporter construct give you enough signal, so that transgenic animals can be identified under the fluorescence stereo microscope.

13. Check your microscope settings. (Is Nomarksi polarization filter removed? Is the light source aligned? Is the light bulb too old?)

14. Use wide rather than narrow filter sets to collect more fluorescent light (you will also get more background fluorescence).

15. Even if the signal looks weak to your eye, it is probably plenty for your camera, so take images and analyze those rather than directly evaluating the fluorescence by eye (use narrow filters for imaging if background fluorescence is a problem). If a sensitive CCD camera on a wide-field microscope detects no signal, there is no signal.

16. GFP as directly fluorescent reporter is less sensitive than other indirect labeling methods, which allow signal enhancement through a second step (antibody staining where secondary antibodies carry the label), or an enzymatic reaction (*lacZ* staining). If you do not see a GFP signal in the fluorescence microscope directly, you can try to detect GFP indirectly with increased sensitivity by processing the animals for immunostaining using an anti-GFP antibody in combination with a secondary labeled antibody.

17. Red fluorophores often give disappointing images at confocal microscopes compared to equally bright green fluorophores for two reasons: photomultiplier detectors are less sensitive in the red part of the spectrum and HeNe lasers for green

excitation are typically weaker than the Argon laser used in the blue part of the spectrum. If you plan to take your samples to the confocal microscope do not combine weak expression with red labels.

18. There are three ways to increase the signal at the confocal microscope. First you can increase the laser power for more excitation light. In general, this also leads to more bleaching and the net effect might even be negative, especially when sampling several focal planes, because your laser will always excite fluorophores in all focal planes. A second option would be to have a larger emission window (wider filter) in order to collect more of the emitted light. Depending on the filters available at your microscope this might not be an option. (**Note**: on the Leica SP2 system the emission window can be freely adjusted because no optical filters are used.) The third option is to open the pinhole, allowing more light to reach the detector. This leads to a loss in confocality because light from other focal planes can now pass through the open pinhole. However, with weak signals you might be able to find a compromise with enough signal and still enough confocality.

19. If your signal still is too weak go back to the wide field microscope. Try deconvolution algorithms to produce "confocal" images.

20. Select your fluorophores to have minimal spectral overlap if possible.

21. The single most effective measure to minimize crosstalk is to prevent excitation of the (second) fluorophore responsible for the crosstalk. To this end use narrow excitation filters as far away from the excitation maximum of the unwanted dye as possible. Use narrow emission filters to prevent signal from the second dye to be detected in the first channel. **Note**: the normalized spectra of the dyes in solution only give hints as to how much crosstalk can be expected, since the actual amount of crosstalk strongly depends on the relative signal strength and, therefore, can be quite variable for a given pair of fluorophores depending on fluorophore concentration. On a confocal microscope use sequential excitation of the fluorophores and minimal excitation light (laser strength).

22. Because signal strength affects crosstalk dramatically and crosstalk from shorter wavelength into longer wavelength is the more serious problem, you should consider combining the stronger expressing partner with the longer wavelength fluorescent label to minimize crosstalk.

23. With two-photon excitation only the fluorophore at the focal spot is excited, eliminating unwanted fluorescence signals from other focal planes by preventing their generation. A second advantage comes from the use of longer wavelength light for excitation (generally twice the excitation wavelength) leading to less phototoxicity and less scattering in the tissue. Less phototoxicity is an advantage for time-lapse recordings and less scattering allows imaging deep inside tissue samples (several hundred microns). A disadvantage are the high energy densities in the focal spot required to achieve the two-photon effect and a rather broad excitation band leading to the simultaneous excitation of all major GFP variants and considerable crosstalk when more than one of them is used. Sampling distance in *C. elegans* animals typically is between 20 and 100 μm, distances where two-photon excitation already has a noticeable effect on signal-to-noise ratio and,

therefore, contrast in deeper focal planes. This is of particular relevance for three-dimensional (3D) reconstructions. However, in samples with strong GFP signals this effect is small and satisfactory reconstructions can be made with data from a regular confocal microscope. Attenutation of excitation light and in particular the use of a spinning disk confocal microscope are alternatives for time-lapse recordings where light induced toxicity is an issue.

24. Open source software for image processing: a large number of software packages for the aquisition and processing of fluorescence images are available. One open-source software project, which has been developed over many years and consequently has a growing number of feature available is ImageJ (*see* http://rsb. info.nih.gov/ij/). Programmed in Java it is a crossplatform software available for all major computer operating systems.

25. Deconvolution: mathematical algorithms can be used to restore the real image from the actual "distorted" image taken at the microscope. The algorithm uses information on how a point source of light is displayed by the microscope taking into account the optical limitations of the system (using a point spread function, ideally measured with fluorescent beads under actual recording conditions). Deconvolution algorithms can be used with wide field images to remove the contribution of out-of-focus light ("blur," "haze"), ideally producing images comparable to confocal images. Generally these algorithms are time consuming and require high-quality, high-resolution data as input. Deconvolution can also be used in combination with confocal images to improve resolution in *z*-axis and signal-to-noise ratio. This is relevant for subsequent 3D reconstructions, which will benefit from deconvolution.

26. Contrast enhancement: digital detection systems have a rather poor dynamic range compared with photographic film (8–12 bit corresponding to 256–4096 gray values for a black and white image). Consequently it is sometimes difficult to adequately display weak signals together with strong signals in a single image. Playing with contrast and brightness usually drives strong signals into saturation when weak signals are enhanced or lets weak signals disappear when strong signals are displayed adequately. Sometimes a compromise can be found with non-linear changes across the dynamic range effectively using different contrast and brightness changes for weak and strong signals (**Fig. 1**). Unfortunately not all programs allow such nonlinear modifications (Adobe® Photoshop® being one of them).

27. 3D reconstructions: several software packages contain modules suitable for 3D display of image stacks. Typically surface rendering algorithms are used to generate a 3D reconstruction of the sample that can be rotated freely in space. Only high-quality images with excellent signal-to-noise ratio and high sampling density are suitable as input for these alrogithms. Even with optimal images fine structures tend to disappear after rendering and are difficult to visualize in this way (*see* **Fig. 2**). 3D images are useful as illustration for a nonexpert audience but are dispensable for scientific evaluation. The experienced observer can access and judge all the relevant information in the original data stack with a
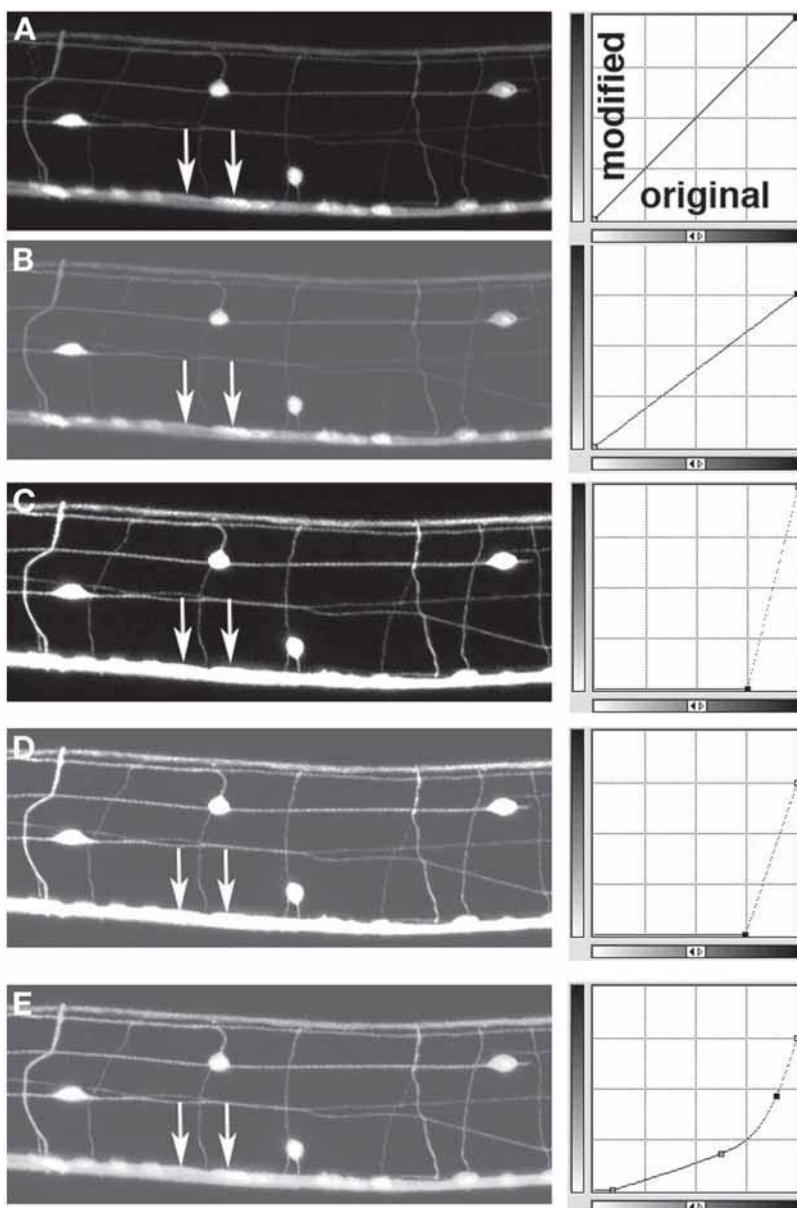
Fig. 1. Nonlinear contrast enhancement. *Caenorhabditis elegans* neurons expressing green fluorescent protein (GFP) with bright cell bodies and rather dim neuronal processes (panneuronal GFP expression, strain NW1229). **(A)** Original image. **(B)** Brightness changed. **(C)** Contrast changed. **(D)** Brightness and contrast changed. **(E)** Brightness and contrast changed nonlinearly, allowing adequate visualization of thin processes and at the same time still some discrimination within brighter regions (arrows, compare to **D**).
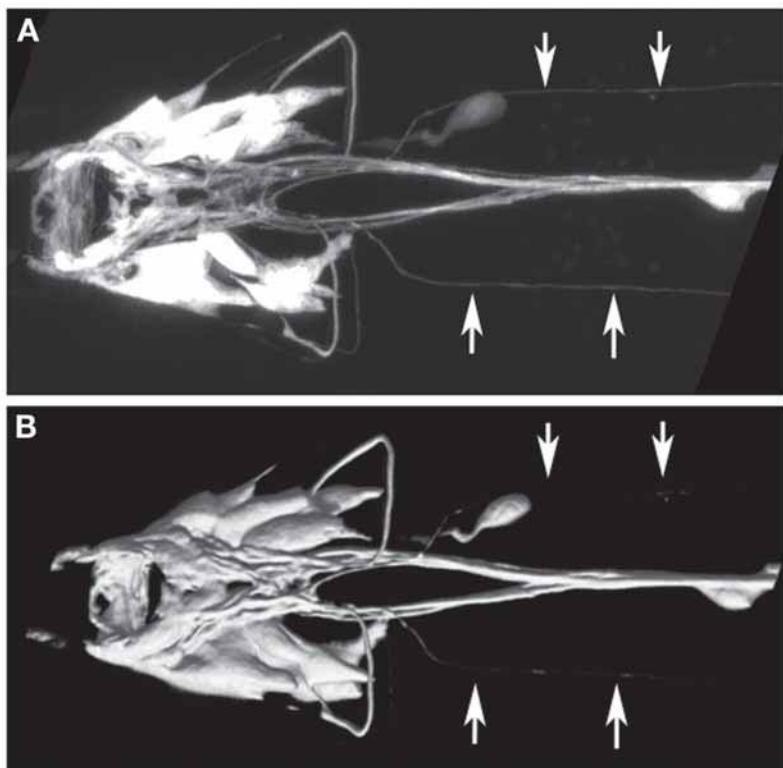
Fig. 2. Three-dimensional (3D) reconstruction and surface rendering. *Caenorhab-ditis elegans* head region, glr-1:green fluorescent protein (GFP) expression. **(A)** Maximum intensity projection of the original data stack. **(B)** Cell surface rendering for 3D reconstruction. Fine structures like subventral axons (arrows) tend to get lost in rendered images, which no longer allow a discrimination of finer structures, which either blend into each other or disappear altogether.

cross-section viewer, a routine display mode available in all imaging software packages.

References

1. Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., and Prasher, D. C. (1994) Green fluorescent protein as a marker for gene expression. *Science* **263,** 802–805.
2. Cubitt, A.B., Heim, R., Adams, S. R., Boyd, A. E., Gross, L. A., and Tsien RY. (1995) Understanding, improving and using green fluorescent proteins. *Trends Biochem. Sci.* **20,** 448–455.
3. Heim, R. and Tsien, R. Y. (1996) Engineering green fluorescent protein for

improved brightness, longer wavelengths and fluorescence resonance energy transfer. *Curr. Biol.* **6,** 178–182.

4. Ormo, M., Cubitt, A. B., Kallio, K., Gross, L. A., Tsien, R. Y., and Remington, S. J. (1996) Crystal structure of the Aequorea victoria green fluorescent protein. *Science* **273,** 1392–1395.

5. Matz, M.V., Fradkov, A. F., Labas, Y. A., et al. (1999) Fluorescent proteins from nonbioluminescent Anthozoa species. *Nat. Biotechnol.* **17,** 969–973.

6. Zhang, S., Ma, C., and Chalfie, M. (2004) Combinatorial marking of cells and organelles with reconstituted fluorescent proteins. *Cell* **119,** 137–144.

7. Ghosh, I., Hamilton, A. D., and Regan, L. (2000) Antiparallel leucine zipper-directed protein reassembly: application to green fluorescent protein. *J. Am. Chem. Soc.* **122,** 5658–5659.

8. Nagai, T., Ibata, K., Park, E. S., Kubota, M., Mikoshiba, K., and Miyawaki, A. (2002) A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nat. Biotechnol.* **20,** 87–90.

9. Wood, W. B. (1998) *The Nematode* Caenorhabditis elegans, *Vol. 17,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

10. Shelton, C. A. and Bowerman, B. (1996) Time-dependent responses to glp-1-mediated inductions in early *C. elegans* embryos. *Development* **122,** 2043–2050.

11. Oegema, K., Desai, A., Rybina, S., Kirkham, M., and Hyman, A. A. (2001) Functional analysis of kinetochore assembly in *Caenorhabditis elegans. J. Cell. Biol.* **153,** 1209–1226.

12. Mohler, W.A. and White, J. G. (1998) Stereo-4-D reconstruction and animation from living fluorescent specimens. *Biotechniques* **24,** 1006–1012.

13. Raich, W.B., Agbunag, C., and Hardin, J. (1999) Rapid epithelial-sheet sealing in the *Caenorhabditis elegans* embryo requires cadherin-dependent filopodial priming. *Curr. Biol.* **9,** 1139–1146.

14. Kramer, J. M., French, R. P., Park, E. C., and Johnson, J. J. (1990) The *Caenorhabditis elegans rol-6* gene, which interacts with the sqt-1 collagen gene to determine organismal morphology, encodes a collagen. *Mol. Cell. Biol.* **10,** 2081–2089.

15. Granato, M., Schnabel, H., and Schnabel, R. (1994) pha-1, a selectable marker for gene transfer in *C. elegans. Nucleic Acids Res.* **22,** 1762–1763.

16. Miller, D. M., Shen, M. M., Shamu, C. E., et al. (1992) *C. elegans unc-4* gene encodes a homeodomain protein that determines the pattern of synaptic input to specific motor neurons. *Nature* **355,** 841–845.

17. Clark, S. G., Lu, X., and Horvitz, H. R. (1994) The *Caenorhabditis elegans* locus lin-15, a negative regulator of a tyrosine kinase signaling pathway, encodes two different proteins. *Genetics* **137,** 987–997.

18. Huang, L. S., Tzou, P., and Sternberg, P. W. (1994) The lin-15 locus encodes two negative regulators of *Caenorhabditis elegans* vulval development. *Mol. Biol. Cell* **5,** 395–411.

13

Electrophysiological Analysis
of Neuronal and Muscle Function in *C. elegans*

Michael M. Francis and Andres Villu Maricq

Summary

The nematode *Caenorhabditis elegans* provides numerous experimental advantages for the identification and characterization of genes required for the function of the nervous system. These advantages include forward and reverse genetic tractability, a relatively simple body plan with an invariant cellular lineage, and a fully sequenced and well-annotated genome. However, one limitation of *C. elegans* is the relative scarcity of electrophysiological data from excitable cells. To address this limitation, high-resolution cellular techniques for probing the roles of specific gene products in the *C. elegans* nervous system have been recently developed. This chapter will provide an overview of the technical requirements for patch-clamp electrophysiological analysis of *C. elegans* neurons and muscle cells, as well as provide some illustrative examples of insights gained from the pairing of electrophysiological techniques with molecular and genetic analysis.

Key Words: *Caenorhabditis elegans*; electrophysiological techniques; patch-clamp; synapse; neurotransmission.

1. Introduction

The nematode *Caenorhabditis elegans* is considered an ideal organism for the identification and characterization of genes contributing to the development and function of the nervous system. Transparent, only 1 mm long, and with a life cycle of approx 4 d, *C. elegans* is easily cultivated in the lab and amenable to light microscopy studies. The nervous system of the hermaphroditic *C. elegans* contains 302 neurons and the location and connectivity of each neuron has been reconstructed from serial section electron micrographs (*1*). The powerful genetics of *C. elegans*, along with its well-described nervous system and repertoire of simple and complex behaviors, has enabled the dissection of behavior at the level of individual genes, identified neurons and sub-

sets of neural circuits *(2)*. However, one perceived limitation has been the relative scarcity of electrophysiological studies. Thus, for most neurons, we do not yet know the identities or properties of the cell-intrinsic, voltage-dependent conductances. We also know little about the synaptic inputs to a given neuron and how synaptic communication is processed by postsynaptic neurotransmitter receptors. To address these shortcomings, high-resolution cellular techniques for probing the roles of specific gene products in the *C. elegans* nervous system have been recently developed. In particular, techniques for electrophysiological analysis of excitable cells have provided important new mechanistic insights into the workings of the *C. elegans* nervous system *(3)*. This chapter will provide an overview of the technical requirements for patch-clamp electrophysiological analysis of *C. elegans* neurons and muscle cells as well as provide some illustrative examples of insights gained from the pairing of electrophysiological techniques with molecular and genetic analysis.

2. Electrophysiological Analysis of the Worm Pharynx

A number of electrophysiological studies in *C. elegans* have made use of two specialized techniques to study the mechanisms underlying rhythmic contractions of the worm pharynx during feeding. The first technique, and most commonly used, is an extracellular recording technique similar to suction electrode recordings (electropharyngeograms [EPGs]) *(4,5)*. EPGs measure the extracellular current flow that is a secondary consequence of rhythmic changes in the muscle transmembrane potential during pharyngeal contractions. Mutants with defects in presynaptic release proteins *(6–9)*, ion channels *(10–12)*, and gap junction proteins *(13)* have all been characterized using EPG recordings. A second technique to measure the activity of the pharyngeal muscle relies on recording the transmembrane potential using an intracellular sharp electrode *(14)*. Sharp electrode recordings have been used to examine the roles of specific ion channel subtypes in pharyngeal muscle action potentials *(15,16)* and neuropeptide signaling *(17)*. Although each of these techniques has proven to be of considerable value in studying cellular excitability in the worm, their application is restricted to the study of the pharynx and the cells that innervate the pharynx, limiting their utility for studying other elements of the nervous system.

3. Patch-Clamping: An Overview

Patch-clamp techniques enable the highest resolution studies of the electrical activity of neurons and muscles. Because it is less injurious to cells than sharp electrode recording techniques, it is particularly useful for studying the electrical properties of small neurons. By generating a high-resistance seal between a glass patch pipet and the cell membrane, one can gain electrical access to cells and monitor changes in electrical activity resulting from ionic currents mediated

by voltage- and ligand-gated ion channels. The technique is extremely sensitive, and in many cases can detect the pA-sized ionic current passing through a single ion channel. Patch-clamp recording techniques were first developed nearly 30 yr ago *(18)*, but were successfully applied to *C. elegans* only recently *(19,20)*. The process of patch-clamp recording from a cell is now rather standard and patch-clamping is routinely used to study conductances in vertebrate, invertebrate, and even plant cells *(21)*. Why then did it take so long for *C. elegans* researchers to exploit this technique? In large part, the answer lies in the particular challenges provided by the anatomy and size of *C. elegans*. The worm is only about 1 mm in length. A tough cuticle surrounds the worm and maintains a high internal hydrostatic pressure. Piercing of the cuticle results in a rapid release of pressure and extrusion of tissue, potentially damaging cells and severing neural connections. Additional challenges include identifying the neuron of interest and gaining electrical access to cell bodies, which are small compared with the cells encountered in most invertebrate and vertebrate preparations. Although body wall muscles are approx 50 µm in length and can be visualized and accessed with relative ease, neuronal cell bodies average only 2–3 µm in diameter and require special measures for identification of the cell(s) of interest as well as for gaining electrical access. In recent years, techniques for overcoming each of these obstacles have been developed. For example, using cell-specific promoters, fluorescent markers such as green fluorescent protein *(22)* can be selectively expressed in muscles or specific neurons of transgenic worms. This genetic strategy has enabled reproducible recording from specific neurons *(23–25)*. Likewise, the adaptation of techniques for permeabilizing cell membranes *(24)* and the development of techniques for fabrication of low-resistance patch pipets *(26)* has facilitated electrical access to even the smallest of neurons. With each new innovation, more researchers have turned to electrophysiological analysis to complement the more traditional tools of the *C. elegans* neurobiology field.

To study the roles of specific gene products in neural signaling, the ability to monitor signaling events in the context of an intact or semi-intact nervous system is of obvious advantage. To this end, several dissection methods for exposing neurons *(19,24)* and muscles *(20)* have been developed. In general, these dissection techniques partially circumvent the anatomical challenges previously discussed, as well as preserve neural connectivity.

3.1. Slit-Worm Dissection

The first preparation for patch-clamp analysis of excitable cells in *C. elegans* was described by Goodman and Lockery *(19)*. In their approach, worms are glued in place and a small incision is made in the cuticle, allowing a "bouquet" of neurons to emerge. With the aid of a green fluorescent protein marker, individual neurons in this bouquet can be easily identified for recording. Slight

modifications have been made to this approach over the past several years in order to extend patch-clamp analysis to *C. elegans* muscle cells *(20)*, specific interneurons of the locomotory control circuit *(24)*, and touch receptor neurons *(27)*.

Each approach requires that the worm be immobilized prior to dissection and recording. Worms are glued down on glass cover slips coated with silicone elastomer (Sylgard 184; Dow Corning) using tissue adhesive. Cyanoacryl glue (www.glustitch.com) rapidly polymerizes on contact with water and works well for this purpose. Glue is applied by mouth pipet using a glass pipet that has been broken back to increase the flow of glue to a suitable rate. Worms can either be incubated on ice to slow their movement prior to gluing or lassoed by applying glue to the head or tail and subsequently glued in place for recording. Slowing down worm movement prior to gluing permits more precise application of glue while lassoing a moving worm alleviates any concerns about temperature effects. Typically, the worm is lying on its right side (preserving right-sided commissural processes) and glue is applied along the dorsal aspect of the worm, taking care not to allow overflow to the area that will be exposed for recordings. A puncture in the cuticle away from the site of recording is then made to relieve hydrostatic pressure. For recording from body wall muscles, the ventral muscle quadrants and ventral cord are exposed by a longitudinal incision along the glue line on the left dorsal side of the worm extending approximately from the vulva to the terminal bulb of the pharynx (**Fig. 1**). A sharp glass needle works well for this purpose. The intestine and gonad are removed using either a glass needle or by suction and the free flap of cuticle created by the incision is then glued to the sylgard-coated cover slip, exposing the ventral musculature for recording. Following muscle exposure, the preparation is washed briefly (~30 s) with a collagenase solution (1 mg/mL in recording solution) to clean the plasma membrane of adherent extracellular proteins and then rinsed with the extracellular recording solution (150 mM NaCl, 5 mM KCl, 4 mM MgCl$_2$, 1 mM CaCl$_2$, 15 mM HEPES, and 10 mM glucose, pH 7.4. Sucrose is added to adjust the osmolarity to 340 mOsm.) Variations on this procedure can be used for recording from head interneurons or touch neurons in the tail. To access these neurons, the worm is glued down in the same fashion as previously described and the area of interest is exposed by dissection. For head interneurons, a transverse incision is made immediately posterior to the terminal bulb and then continued anteriorly along the glue line toward the tip of the nose (**Fig. 1**; **ref. 24**). The resulting triangle of cuticle is glued to the sylgard-coated cover slip, exposing the nerve ring and interneurons for recording. For recording from PLM touch neurons, cell bodies are exposed by a ventral incision along the tail *(27)*.
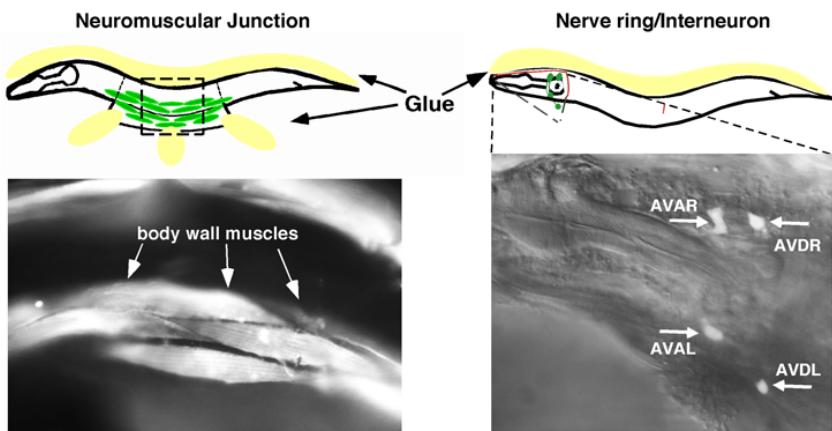
Fig. 1. *Caenorhabditis elegans* preparations for electrophysiology. Left, Dissected preparation for muscle recordings. The box on the sketch marks the area pictured below, in which *C. elegans* is dissected open and the body-wall muscles exposed for patch-clamp recording. Green fluorescent protein (GFP) expression in transgenic worms is limited to muscles using the promoter of the *myo-3* gene *(60)*. Right, the dissection used for exposing the bilaterally symmetrical command interneurons AVA and AVD. These interneurons can be readily identified in transgenic worms that express GFP under the control of the *nmr-1* promoter *(61)*. The markings represent the sites of incision and show the the orientation of the final dissected preparation. The positions of the GFP-positive interneuron cell bodies in the dissected preparation are shown and correspond to the labeled cell bodies in the image below.

3.2. Patch-Clamping in C. elegans

After a successful dissection, the patch-pipet is brought into contact with the cell membrane and the application of mild suction (approx –5 to –10 cm H_2O) causes the cell membrane to enter the pipet-tip opening, forming an omega shape. If all goes well, a high-resistance seal, known as a gigaseal, is produced allowing the experimenter to record the electrical activity of channels in the plasma membrane plugging the pipet tip (on-cell or cell-attached configuration). Alternatively, the underlying membrane can be ruptured, allowing electrical access to the cell interior and recording the electrical activity of the whole cell. These techniques are used, with slight variations, to record from a wide variety of cells *(28)*. No major modifications are required to extend these techniques to the cells of *C. elegans*. For recording from worm body wall muscles, patch pipets are fire-polished to a resistance of 4–7 MΩ. Slight positive pressure is placed on the pipet as it enters the bath solution to prevent contaminating the pipet tip with particles on the surface of the bath solution. Failure to observe this precaution guarantees failure to achieve a high-resistance seal. Once in the

bath, small step changes in voltage are applied to the patch pipet to monitor pipet resistance. The patch pipet is brought in close proximity to the cell body of interest and slowly advanced until the pipet resistance increases. At this point, positive pressure is released and a seal between the membrane and the glass pipet begins to form, slight negative pressure will aid in seal formation. After forming a high resistance seal—typically 10–50 GΩ—increased negative pressure coupled with the membrane "zap" function on the amplifier achieves whole-cell access, signified by a rapid increase in the slow capacitive transient observed in response to steps in the pipet voltage. Recording from neurons follows the same steps as described for muscle. However, obtaining whole-cell access to neurons requires more extravagant measures. Because the neuronal cell bodies are small, high-resistance patch pipets (8–10 MΩ; smaller tip opening) must be used so that the cell body is not inadvertently sucked into the pipet. Unfortunately, the use of high-resistance patch pipets dramatically decreases one's ability to obtain whole-cell access. Two strategies have been devised to circumvent this problem. The adaptation of techniques used for generating permeabilized patches permits improved whole-cell access using conventionally shaped patch pipets *(24)*. Addition of the antibiotic nystatin (25 µg/mL) to the intracellular pipet solution significantly increases the probability of obtaining whole-cell access within a few minutes of obtaining a seal. Alternatively, low-resistance patch pipets with small tip openings can be produced by the technique of pressure polishing *(26)*. Although these pipets obviate the need for nystatin in the recording pipet, their production requires a specialized piece of equipment for pressure polishing. Once whole cell access is achieved, the experimenter can clamp the transmembrane potential of the cell by modifying the voltage inside the pipet (whole-cell voltage-clamp), and simultaneously monitor the electrical current flowing into or out of the cell in response to changes in voltage or exposure to drugs or neurotransmitters. Alternatively, the current-clamp configuration allows one to monitor changes in the cell membrane potential as a function of channel opening.

4. The *C. elegans* Neuromuscular Junction:
A Model Polyinnervated Synapse

In *C. elegans*, specialized projections from the body wall muscle, called muscle arms, extend to the ventral nerve cord where they make *en passant* synapses with cholinergic and GABAergic neuronal processes. Patch-clamp recordings from *C. elegans* muscles have revealed a variety of ligand- and voltage-gated currents, including acetylcholine (ACh)- and GABA-gated ionotropic receptors as well as voltage-gated K^+ and Ca^{2+} currents *(20,29,30)*. Electrophysiological analysis of mutants with disrupted neuromuscular function has helped characterize gene products that contribute to synaptic transmission.
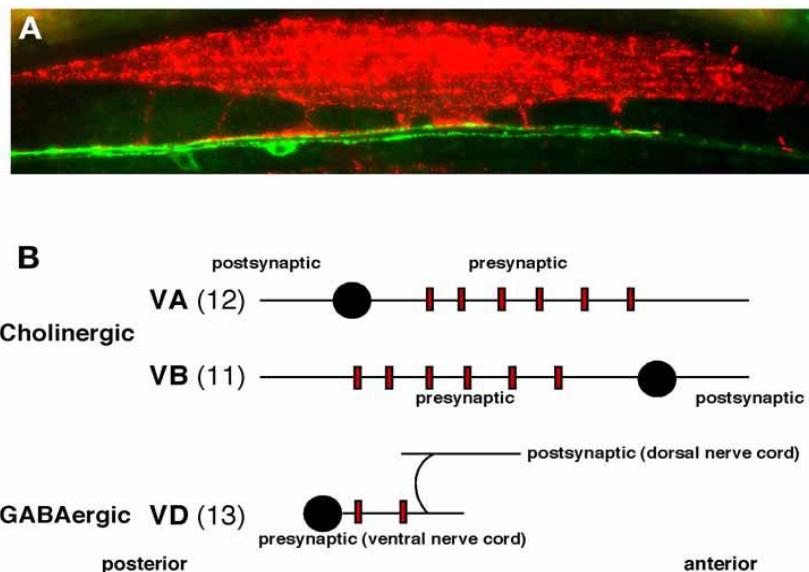
Fig. 2. Body wall muscles are innervated by multiple classes of motor neurons. **(A)** Image of body wall muscle and nerve cord from a transgenic worm expressing dsRed in the body wall muscle using the *myo-3* promoter, and green fluorescent protein in the ventral nerve cord using the *unc-4* promoter *(62)*. Muscle arms extend from the muscle cell body to make synaptic contact with motor neuron processes of the ventral nerve cord. **(B)** Schematic of motor neuron cell types (VA, VB, and VD) that have presynaptic release sites (rectangles) at neuromuscular synapses of the ventral nerve cord. The number of neurons in each classification is indicated in parentheses *(1)*. Rectangles do not reflect the actual number of release sites.

Examples include the *egl-19* gene, which encodes an L-type voltage-gated Ca^{2+} channel subunit required for voltage-activated Ca^{2+} currents in muscle cells *(31)*, and the *slo-2* gene, which encodes a delayed rectifier potassium channel that is a major contributor to the prolonged, steady-state component of K^+ current found in body wall muscles *(29,32)*. *C. elegans* body wall muscles are innervated by both GABAergic and cholinergic motor neurons (**Fig. 2**) and the postsynaptic receptor fields on *C. elegans* muscle include at least three classes of ligand-gated receptors *(20,33,34)*. The *unc-49* gene encodes multiple, alternatively spliced subunits of a GABA-activated Cl^- channel *(35,36)*, whereas subunits comprising two classes of ACh-gated receptors are encoded by a number of genes. The *unc-38*, *unc-63*, *unc-29*, *lev-1*, and *lev-8* genes all contribute to a class of ACh receptor that exhibits slow kinetics and can be activated by the agonist levamisole (levamisole receptor *[20,36a,37–40]*), whereas the *acr-16* gene is essential for fast synaptic currents that can be activated by the agonist nicotine *(37)*.

Many of the previously mentioned studies used pressure perfusion of agonists to activate specific classes of ligand-gated ion channels in voltage-clamped muscle cells. In this technique, a patch pipet mounted on a micromanipulator and filled with the appropriate chemical diluted in recording solution is brought in close proximity to the voltage-clamped cell and a timed step increase in pressure on the pipet is used to drive the chemical out of the pipet tip and onto the cell of interest. This technique is useful because it is relatively uncomplicated and measures the activity of both synaptic and extrasynaptic ligand-gated ion channels. However, drugs cannot be pressure-applied very rapidly, so currents that quickly desensitize in the continued presence of drug often cannot be reliably recorded. Moreover, the time course of current responses to drug application can vary widely as a function of channel localization and placement of the agonist application pipet, leading to erroneous conclusions about channel behavior; hence, it is wise to sample multiple regions of the muscle or neuron of interest. Although this technique is very useful for studying ion channel function, it is not particularly informative about the workings of the synapse itself. Synaptic transmission can be more directly studied by recording endogenous and evoked synaptic transmission in various mutant backgrounds.

4.1. Measuring Synaptic Currents at the Neuromuscular Junction

Endogenous synaptic currents can be recorded from *C. elegans* muscles with relative ease because the neuromuscular synapses are typically not disrupted by the dissection. Currents mediated by specific receptor subtypes can be studied in isolation of other currents by using available mutations in genes encoding ion channel subunits, pharmacological antagonists, and by manipulating extracellular or intracellular ion composition. Under typical recording conditions, *C. elegans* neuromuscular synapses appear tonically active, perhaps because neurons are maintained at a relatively depolarized potential (J. Mellem, personal communication). Because of the relatively high rate of endogenous synaptic activity, it is straightforward to rapidly gather detailed information on synaptic event frequency and amplitude for specific receptor subtypes. This technique is of obvious utility for characterizing mutants with deficiencies in postsynaptic receptor field organization or neurotransmitter release. For example, *acr-16* mutant worms lack a specific class of large-amplitude endogenous cholinergic synaptic events at the neuromuscular junction (NMJ *[37]*). Similarly, a variety of mutations in the neurotransmitter release machinery result in a reduced frequency of endogenous synaptic events *(41–44)*.

Nerve-evoked currents can also be recorded from *C. elegans* muscles (**Fig. 3**). Typically, a stimulating pipet is placed on the ventral nerve cord approx 1–2 muscle-lengths anterior of the voltage-clamped muscle. Notably, evoked currents recorded in this configuration appear predominantly cholinergic, perhaps
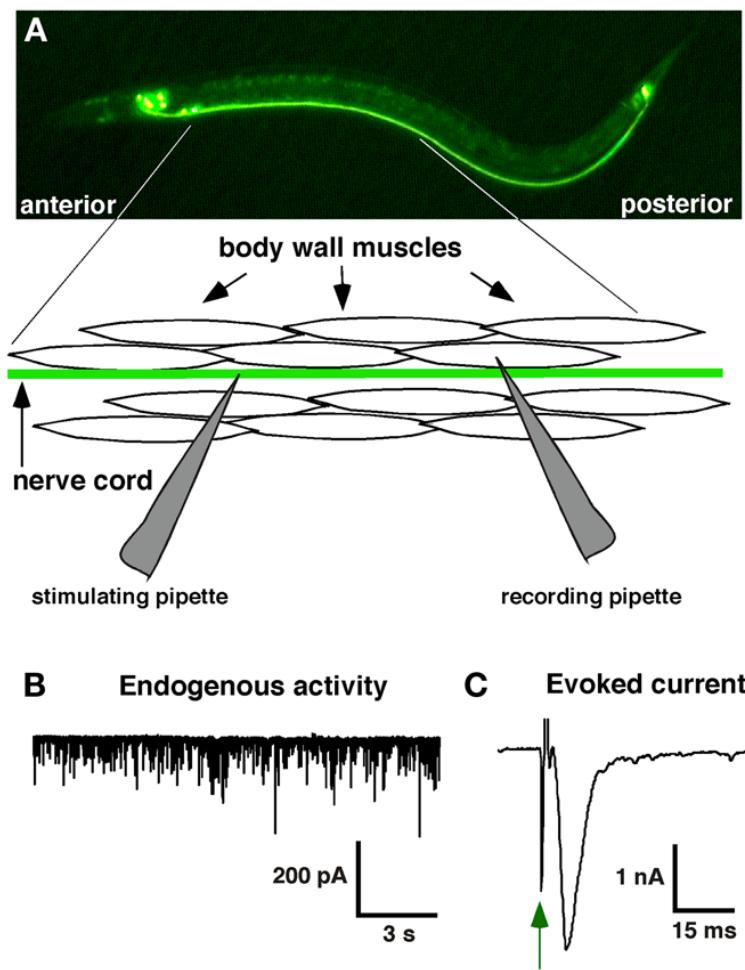
Fig. 3. Synaptic currents at the *Caenorhabditis elegans* neuromuscular junction (NMJ). **(A)** Confocal image of a transgenic worm expressing *nmr-1::green fluorescent protein (GFP)* (top), and schematic of preparation for recording evoked currents from *C. elegans* muscle cells (bottom). Expression a GFP reporter in the ventral nerve cord facilitates pipet placement for recording of evoked synaptic currents. **(B)** Endogenous synaptic activity at the NMJ recorded from a wild-type worm. **(C)** Evoked synaptic current at the NMJ recorded from a wild-type worm. The arrow indicates the stimulus artifact.

because GABAergic VD motor neurons extend more localized projections (**Fig. 2**) compared with cholinergic motor neurons. A brief (~1 ms), large (10–40 V) depolarizing stimulus is applied to the ventral cord while simultaneously recording the evoked current in the voltage-clamped muscle cell.

Depolarization of the ventral cord is marked by the presence of a large stimulus artifact and leads to the rapid release of neurotransmitter from motor neurons and activation of postsynaptic receptors. One limitation of this technique is the inability to activate specific synaptic inputs. Because an extracellular stimulus is employed, it has not been possible to produce focal depolarization of individual neuronal cell bodies. Thus, the nerve-evoked currents are likely produced by concerted depolarization of groups of neuronal processes. Likewise, because pipet placement varies somewhat with each preparation, it is difficult to reproducibly modify stimulus intensity using this type of extracellular stimulation. Thus, most experiments to date have simply assessed maximal neurotransmitter release. Nonetheless, this technique has been extremely useful in characterizing genes that play a role in regulating neuromuscular transmission.

4.2. Analysis of ACh Receptors at the NMJ

Several recent studies have used electrophysiological analysis of the NMJ in conjunction with molecular and cellular techniques to characterize gene products required for the localization and maintenance of postsynaptic receptors. As synapses on the body wall muscles include multiple classes of postsynaptic receptors, the NMJ is an ideal preparation for studying mechanisms of trafficking and segregation of postsynaptic receptors to cellular domains, as well as synaptic microdomains. The *lev-10* and *cam-1* genes encode proteins selectively involved in the localization of either levamisole receptors (LevRs) or ACR-16 receptors, respectively *(37,45)*. LevRs normally localize in distinct clusters at the junction of the ventral nerve cord with body wall musculature and are required for normal worm movement. The *lev-10* gene encodes a novel CUB domain protein that has recently been implicated in clustering LevRs. In *lev-10* mutants, LevRs no longer cluster opposite presynaptic release sites and instead are diffusely distributed across the muscle cell surface. Interestingly, responses to exogenous application of levamisole are normal in *lev-10* mutants, indicating that surface expression of LevRs is not affected. In contrast, nerve-evoked responses are substantially decreased, consistent with reduced synaptic localization of LevRs. Synaptic currents mediated by ACR-16 receptors are dependent on the *cam-1* gene product. The *cam-1* gene encodes a Ror receptor tyrosine kinase and ACR-16 receptors are mislocalized in *cam-1* mutants. The localization and function of LevRs is unaffected by mutation of the *cam-1* gene, consistent with a specific role for CAM-1 in regulating ACR-16 receptors.

4.3. Analysis of Neurotransmitter Release at the NMJ

Identifying the molecular machinery required for vesicular release of neurotransmitter is of central importance to a detailed understanding of synaptic

transmission. Recent work in *C. elegans* has made significant contributions toward this goal and has been instrumental in establishing distinct roles for gene products at specific points in the vesicle cycle. For example, functional roles have been described for the *unc-10*, *unc-13*, *unc-18*, *unc-26* (synaptojanin), and *unc-57* (endophilin) gene products in vesicular release and endocytosis *(41– 44,46)*. Electrophysiological analysis of each of these mutants has revealed decreases in both endogenous event frequency and evoked current amplitude. How then does electrophysiological analysis help assign particular roles to these molecules in the synaptic vesicle cycle? Over time, a number of protocols for testing specific aspects of synaptic release have been developed for vertebrate preparations and several of these have been adapted for use in *C. elegans*. For example, mutants with defects in vesicle endocytosis might be expected to fatigue rapidly with repeated stimulation because of decreased vesicle recy- cling. Fatigue analysis was used to show that synaptic responses of *unc-26* and *unc-57* mutants fatigue rapidly and to comparable levels *(41)*. Furthermore, the rate of fatigue was not enhanced in *unc-26; unc-57* double mutants, suggesting that these proteins function in the same endocytic pathway. Typically, an initial baseline level of release in response to nerve stimulation is established and then the amplitude of evoked current to repeated stimuli is measured. Usually, depo- larizing stimuli are applied in trains of 10 at a rate of 0.5 Hz and the decay in evoked current is plotted as a function of the current response to the initial stimu- lus. Similar analyses have been employed to characterize clearance of neu- rotransmitter from the synaptic cleft. The *snf-6* gene encodes a transporter that influences synaptic transmission at the NMJ directly by mediating the reuptake of ACh *(47)*. *snf-6* mutants show enhanced evoked current responses to repeated stimulation, presumably as a result of altered ACh reuptake.

5. Electrophysiological Analysis of *C. elegans* Neurons

In addition to analysis of synaptic currents at the NMJ, a number of recent studies have begun to examine voltage- and ligand-gated receptors in identi- fied neurons. In vivo patch-clamp recordings have revealed robust ligand-gated currents in response to glutamate in the interneurons AVA and AVD *(23–25)*, as well as voltage-activated potassium currents in the neurons ASE and CEP *(19,48,49)*.

Pressure application of glutamate to the AVA interneuron has revealed two major current components, a large and rapid current component that is dependent on the GLR-1 and GLR-2 AMPA receptor subunits *(23)*, and a smaller yet longer-lasting current. The fast, GLR-1-dependent current medi- ates the backing response to nose touch and hyperosmotic stimuli *(23, 50,51)*. The slower current is activated by the agonist *N*-methyl-D-aspartate and is abolished by a deletion mutation in the NMR-1 subunit *(52)*. Interest-

ingly, GLR-1 mediated currents are dependent on the CUB domain protein SOL-1 (*25*). *sol-1* mutants specifically lack fast glutamate-gated currents and phenocopy the behavior of *glr-1* mutants. Based on coimmunoprecipitation experiments, SOL-1 and GLR-1 appear to physically interact. Because GLR-1 receptors show normal surface expression and localization in the neurons of *sol-1* mutants, SOL-1 is likely required for the function of GLR-1 receptors.

5.1. Primary Sensory Neuron Recordings

Although it is now straightforward to record ligand- and voltage-gated currents from identified neurons, it is much more difficult to study nerve-evoked currents or endogenous synaptic activity in the nervous system. Although endogenous synaptic activity can be clearly observed in *C. elegans* neurons, the number of synaptic inputs to a particular cell complicates analysis. Likewise, the study of evoked synaptic currents in neurons requires new methodologies to selectively stimulate specific synaptic inputs. Recently, the receptor potential in touch-cell neurons was analyzed by patch-clamp recording from the PLM sensory neurons while mechanically stimulating the cuticle (*27*). A flexible glass probe mounted on a piezo-electric bimorph was pressed against the body wall to generate touch responses. Transient "on" and "off" mechanoreceptor current responses were observed, indicating that *C. elegans* touch receptors respond to changes in force rather than steady-state pressure. Interestingly, although worms show behavioral habituation to repeated touches, cellular responses to repeated mechanical stimuli remained stable over time, suggesting that adaptation does not occur at the level of the sensory neuron. Electrophysiological analysis of *mec* mutant worms—previously isolated in screens for defective mechanosensation (*53*)—confirmed that *mec-2*, *mec-4*, *mec-6*, *mec-7*, and *mec-10* all contribute to the machinery required for transducing mechanical signals in *C. elegans*.

5.2. Patch-Clamp Analysis of Cultured C. elegans Cells

The recent development of techniques for culturing *C. elegans* cells has further increased accessibility of the worm for electrophysiological analysis (*54*). Although synaptic currents have not yet been demonstrated in cultured *C. elegans* neurons, patch-clamp recording of cultured cells has allowed for the rapid characterization of membrane currents without the need for specialized dissection techniques. For example, a recent study has demonstrated the presence of two distinct voltage-independent calcium conductances in cultured *C. elegans* intestinal epithelial cells (*55*), suggesting that these channels may play a role in regulating Ca^{2+}-dependent stereotyped behaviors, such as the defecation motor program (*56*). Similarly, recent electrophysiological analysis of cul-

tured *C. elegans* dopaminergic neurons revealed depolarizing currents associated with dopamine transporter activity, suggesting that dopamine transporters can adopt a transient channel-like configuration *(57)*. In addition to conventional mutant analysis, other techniques for knockdown of gene expression such as RNA interference can be more effectively applied to cultured neurons than neurons in vivo. This general strategy was used to study the role of the mink-related peptide (MPS) family of K^+ channel ancillary subunits in chemosensory neurons *(58,59)*. The *mps* genes encode single-pass transmembrane domain proteins that co-associate with the KVS-1 K^+ channel subunit. RNAi knockdown of MPS family members altered the current amplitude and kinetic properties of voltage-gated K^+ currents in cultured ASE neurons. RNAi targeting of the same genes in worms produced corresponding alterations in the sensitivity to soluble chemoattractants, suggesting that selective coexpression of the potassium channel subunit KVS-1 with members of the MPS family may serve to tune the excitability of specific chemosensory neurons.

6. Limitations and Future Directions

The unique opportunity to combine genetic perturbation with electrophysiology and behavioral analysis in *C. elegans* has greatly expanded our understanding of ion channel regulation, synaptic development and neurotransmission. However, continued advances will depend on the ongoing development of more refined techniques for analyzing cellular excitability in the worm. Electrical, mechanical, or optical techniques need to be developed to selectively activate neural inputs to identified neurons. The study of evoked synaptic currents is already feasible at the NMJ using extracellular stimulation. However, paired recordings between identified motor neuron–muscle cell pairs may provide for more experimental flexibility in controlling neurotransmitter release and postsynaptic excitation. For example, at present, the analysis of neurotransmission fatigue at the NMJ is complicated by the instability of the preparation over time. Thus, complete recovery from fatigue—as would be expected if this were a purely synaptic phenomenon—is rarely realized. Techniques for more focal stimulation may permit analysis of the time course of recovery from fatigue as well as provide a convenient internal control for these kinds of experiments. Similarly, the study of evoked currents in the nervous system has thus far been limited to primary sensory neurons where external stimulation can be used to elicit synaptic potentials in subsets of neurons. Paired neuronal recordings may provide a means for studying neuronal control of second- and third-order neurons that receive multiple synaptic inputs. Continued development of new stimulation techniques and their application to interneurons and motor neurons bodes well for a mechanistic understanding of the *C. elegans* nervous system.

A second major hurdle is the lack of a well-developed pharmacology. In many cases, cells of interest are innervated by multiple presynaptic neurons and express a variety of postsynaptic receptor subtypes that need to be isolated using pharmacological agents. Specific drugs—agonists and antagonists—can be used to acutely perturb synaptic function in a controlled manner such that the functional roles of specific neuronal inputs and specific neurotransmitter receptors can be explored. Drugs that are useful for the study of the *C. elegans* nervous system are slowly being identified or developed and a combination of genetics and pharmacology will continue to aid our progress in understanding nervous system function.

References

1. White, J. G., Southgate, E., Thomson, J. N., and Brenner, S. (1986) The structure of the nervous system of the nematode *Caenorhabditis elegans*. *Phil. Trans. Roy. Soc. (Lond.) B*, **314,** 1–340.
2. De Bono, M. and Maricq, A. V. (2005) Neuronal substrates of complex behaviors in *C. elegans*. *Annu. Rev. Neurosci.* **28,** 451–501.
3. Francis, M. M., Mellem, J. E., and Maricq, A. V. (2003) Bridging the gap between genes and behavior: recent advances in the electrophysiological analysis of neural function in *Caenorhabditis elegans*. *Trends Neurosci.* **26,** 90–99.
4. Raizen, D. M. and Avery, L. (1994) Electrical activity and behavior in the pharynx of *Caenorhabditis elegans*. *Neuron* **12,** 483–495.
5. Avery, L., Raizen, D., and Lockery, S. (1995) Electrophysiological methods *Methods Cell Biol.* **48,** 251–269.
6. Saifee, O., Wei, L., and Nonet, M. L. (1998) The *Caenorhabditis elegans unc-64* locus encodes a syntaxin that interacts genetically with synaptobrevin. *Mol. Biol. Cell* **9,** 1235–1252.
7. Nonet, M. L., Staunton, J. E., Kilgard, M. P., et al. (1997) *Caenorhabditis elegans rab-3* mutant synapses exhibit impaired function and are partially depleted of vesicles. *J. Neurosci.* **17,** 8061–8073.
8. Nonet, M. L., Saifee, O., Zhao, H., Rand, J. B., and Wei, L. (1998) Synaptic transmission deficits in *Caenorhabditis elegans* synaptobrevin mutants. *J. Neurosci.* **18,** 70–80.
9. Willson, J., Amliwala, K., Davis, A., et al. (2004) Latrotoxin receptor signaling engages the UNC-13-dependent vesicle-priming pathway in *C. elegans*. *Curr. Biol.* **14,** 1374–1379.
10. Lee, R. Y., Lobel, L., Hengartner, M., Horvitz, H. R., and Avery, L. (1997) Mutations in the alpha1 subunit of an L-type voltage-activated Ca^{2+} channel cause myotonia in *Caenorhabditis elegans*. *Embo. J.* **16,** 6066–6076.
11. Dent, J. A., Davis, M. W., and Avery, L. (1997) *avr-15* encodes a chloride channel subunit that mediates inhibitory glutamatergic neurotransmission and ivermectin sensitivity in *Caenorhabditis elegans*. *Embo. J.* **16,** 5867–5879.
12. Wang, Z. W., Saifee, O., Nonet, M. L., and Salkoff, L. (2001) SLO-1 potassium channels control quantal content of neurotransmitter release at the *C. elegans* neuromuscular junction. *Neuron* **32,** 867–881.

13. Starich, T. A., Lee, R. Y., Panzarella, C., Avery, L., and Shaw, J. E. (1996) *eat-5* and *unc-7* represent a multigene family in *Caenorhabditis elegans* involved in cell-cell coupling. *J. Cell Biol.* **134,** 537–548.

14. Pemberton, D. J., Franks, C. J., Walker, R. J., and Holden-Dye, L. (2001) Characterization of glutamate-gated chloride channels in the pharynx of wild-type and mutant *Caenorhabditis elegans* delineates the role of the subunit GluCl-alpha2 in the function of the native receptor. *Mol. Pharmacol.* **59,** 1037–1043.

15. Davis, M. W., Somerville, D., Lee, R. Y., Lockery, S., Avery, L., and Fambrough, D. M. (1995) Mutations in the *Caenorhabditis elegans* Na,K-ATPase alpha-subunit gene, *eat-6*, disrupt excitable cell function. *J. Neurosci.* **15,** 8408–8418.

16. Franks, C. J., Pemberton, D., Vinogradova, I., Cook, A., Walker, R. J., and Holden-Dye, L. (2002) Ionic basis of the resting membrane potential and action potential in the pharyngeal muscle of *Caenorhabditis elegans*. *J. Neurophysiol.* **87,** 954–961.

17. Rogers, C., Reale, V., Kim, K., et al. (2003) Inhibition of *Caenorhabditis elegans* social feeding by FMRFamide-related peptide activation of NPR-1. *Nat. Neurosci.* **6,** 1178–1185.

18. Neher, E. and Sakmann, B. (1976) Single-channel currents recorded from membrane of denervated frog muscle fibres. *Nature* **260,** 779–802.

19. Goodman, M. B., Hall, D. H., Avery, L., and Lockery, S. R. (1998) Active currents regulate sensitivity and dynamic range in *C. elegans* neurons. *Neuron* **20,** 763–772.

20. Richmond, J. E. and Jorgensen, E. M. (1999) One GABA and two acetylcholine receptors function at the *C. elegans* neuromuscular junction. *Nat. Neurosci.* **2,** 791–797.

21. Sakmann, B. and Neher, E. (1995) *Single-Channel Recording, 2nd Ed.,* Kluwer Academic/Plenum Publishers, New York, NY.

22. Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., and Prasher, D. C. (1994) Green fluorescent protein as a marker for gene expression. *Science* **263,** 802–805.

23. Mellem, J. E., Brockie, P. J., Zheng, Y., Madsen, D. M., and Maricq, A. V. (2002) Decoding of polymodal sensory stimuli by postsynaptic glutamate receptors in *C. elegans*. *Neuron* **36,** 933–944.

24. Brockie, P. J., Mellem, J. E., Hills, T., Madsen, D. M., and Maricq, A. V. (2001) The *C. elegans* glutamate receptor subunit NMR-1 is required for slow NMDA-activated currents that regulate reversal frequency during locomotion. *Neuron* **31,** 617–630.

25. Zheng, Y., Mellem, J. E., Brockie, P. J., Madsen, D. M., and Maricq, A. V. (2004) SOL-1 is a CUB-domain protein required for GLR-1 glutamate receptor function in *C. elegans*. *Nature* **427,** 451–457.

26. Goodman, M. B. and Lockery, S. R. (2000) Pressure polishing: a method for re-shaping patch pipettes during fire polishing. *J. Neurosci. Methods* **100,** 13–15.

27. O'Hagan, R., Chalfie, M., and Goodman, M. B. (2005) The MEC-4 DEG/ENaC channel of *Caenorhabditis elegans* touch receptor neurons transduces mechanical signals. *Nat. Neurosci.* **8,** 43–50.

28. Hamill, O. P., Marty, E., Neher, B., Sakmann, B., and Sigworth, F. J. (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv.* **391,** 85–100.
29. Santi, C. M., Yuan, A., Fawcett, G., et al. (2003) Dissection of K+ currents in *Caenorhabditis elegans* muscle cells by genetics and RNA interference. *Proc. Natl. Acad. Sci. USA* **100,** 14,391–14,396.
30. Jospin, M., Mariol, M. C., Segalat, L., and Allard, B. (2002) Characterization of K+ currents using an in situ patch clamp technique in body wall muscle cells from *Caenorhabditis elegans*. *J. Physiol.* **544,** 373–384.
31. Jospin, M., Jacquemond, V., Mariol, M. C., Segalat, L., and Allard, B. (2002) The L-type voltage-dependent Ca^{2+} channel EGL-19 controls body wall muscle function in *Caenorhabditis elegans*. *J. Cell Biol.* **159,** 337–348.
32. Yuan, A., Santi, C. M., Wei, A., et al. (2003) The sodium-activated potassium channel is encoded by a member of the *Slo* gene family. *Neuron* **37,** 765–773.
33. Schuske, K., Beg, A. A., and Jorgensen, E. M. (2004) The GABA nervous system in *C. elegans*. *Trends Neurosci,* **27,** 407–414.
34. Rand, J. B., Duerr, J. S., and Frisby, D. L. (2000) Neurogenetics of vesicular transporters in *C. elegans*. *Faseb. J.* **14,** 2414–2422.
35. Bamber, B. A., Beg, A. A., Twyman, R. E., and Jorgensen, E. M. (1999) The *Caenorhabditis elegans unc-49* locus encodes multiple subunits of a heteromultimeric GABA receptor. *J. Neurosci.* **19,** 5348–5359.
36. Bamber, B. A., Richmond, J. E., Otto, J. F., and Jorgensen, E. M. (2005) The composition of the GABA receptor at the *Caenorhabditis elegan*s neuromuscular junction. *Br. J. Pharmacol.* **144,** 502–509.
36a. Touroutine, D., Fox, R. M., Von Stetina, S. E., Burdina, A., Miller, D. M. 3rd, Richmond, J. E. (2005) *acr-16* encodes an essential subunit of the levamisole-resistant nicotinic receptor at the *Caenorhabditis elegans* neuromuscular junction. *J. Biol. Chem.* **280,** 27,013–27,021.
37. Francis, M. M., Evans, S. P., Jensen, M., et al. (2005) The Ror receptor tyrosine kinase CAM-1 is required for ACR-16 mediated synaptic transmission at the *C. elegans* neuromuscular junction. *Neuron* **46,** 581–594.
38. Fleming, J. T., Squire, M. D., Barnes, T. M., et al. (1997) *Caenorhabditis elegans* levamisole resistance genes *lev-1*, *unc-29*, and *unc-38* encode functional nicotinic acetylcholine receptor subunits. *J. Neurosci.* **17,** 5843–5857.
39. Culetto, E., Baylis, H. A., Richmond, J. E., et al. (2004) The *Caenorhabditis elegans unc-63* gene encodes a levamisole-sensitive nicotinic acetylcholine receptor alpha subunit. *J. Biol. Chem.* **279,** 42,476–42,483.
40. Towers, P. R., Edwards, B., Richmond, J. E., and Sattelle, D. B. (2005) The *Caenorhabditis elegans lev-8* gene encodes a novel type of nicotinic acetylcholine receptor alpha subunit. *J. Neurochem.* **93,** 1–9.
41. Schuske, K. R., Richmond, J. E., Matthies, D.S., et al. (2003) Endophilin is required for synaptic vesicle endocytosis by localizing synaptojanin. *Neuron* **40,** 749–762.
42. Weimer, R. M., Richmond, J. E., Davis, W. S., Hadwiger, G., Nonet, M. L., and Jorgensen, E. M. (2003) Defects in synaptic vesicle docking in *unc-18* mutants *Nat. Neurosci.* **6,** 1023–1030.

43. Richmond, J. E., Davis, W. S., and Jorgensen, E. M. (1999) UNC-13 is required for synaptic vesicle fusion in *C. elegans*. *Nat. Neurosci.* **2,** 959–964.

44. Koushika, S. P., Richmond, J. E., Hadwiger, G., Weimer, R. M., Jorgensen, E. M., and Nonet, M. L. (2001) A post-docking role for active zone protein Rim. *Nat. Neurosci.* **4,** 997–1005.

45. Gally, C., Eimer, S., Richmond, J. E., and Bessereau, J. L. (2004) A transmembrane protein required for acetylcholine receptor clustering in *Caenorhabditis elegans*. *Nature* **431,** 578–582.

46. Richmond, J. E., Weimer, R. M., and Jorgensen, E. M. (2001) An open form of syntaxin bypasses the requirement for UNC-13 in vesicle priming. *Nature* **412,** 338–341.

47. Kim, H., Rogers, M. J., Richmond, J. E., and McIntire, S. L. (2004) SNF-6 is an acetylcholine transporter interacting with the dystrophin complex in *Caenorhabditis elegans*. *Nature* **430,** 891–896.

48. Davies, A. G., Pierce-Shimomura, J. T., Kim, H., et al. (2003) A central role of the BK potassium channel in behavioral responses to ethanol in *C. elegans*. *Cell* **115,** 655–666.

49. Pierce-Shimomura, J. T., Faumont, S., Gaston, M. R., Pearson, B. J., and Lockery, S. R. (2001) The homeobox gene *lim-6* is required for distinct chemosensory representations in *C. elegans*. *Nature* **410,** 694–698.

50. Maricq, A. V., Peckol, E., Driscoll, M., and Bargmann, C. I. (1995) Mechanosensory signalling in *C. elegans* mediated by the GLR-1 glutamate receptor. *Nature* **378,** 78–81.

51. Hart, A. C., Sims, S., and Kaplan, J. M. (1995) Synaptic code for sensory modalities revealed by *C. elegans* GLR-1 glutamate receptor. *Nature* **378,** 82–85.

52. Brockie, P. J., Mellem, J. E., Hills, T., Madsen, D. M., and Maricq, A. V. (2001) The *C. elegans* glutamate receptor subunit NMR-1 is required for slow NMDA-activated currents that regulate reversal frequency during locomotion. *Neuron* **31,** 617–630.

53. Huang, M. and Chalfie, M. (1994) Gene interactions affecting mechanosensory transduction in *Caenorhabditis elegans*. *Nature* **367,** 467–470.

54. Christensen, M., Estevez, A., Yin, X., et al. (2002) A primary culture system for functional analysis of *C. elegans* neurons and muscle cells. *Neuron* **33,** 503–514.

55. Estevez, A. Y., Roberts, R. K., and Strange, K. (2003) Identification of store-independent and store-operated Ca^{2+} conductances in *Caenorhabditis elegans* intestinal epithelial cells. *J. Gen. Physiol.* **122,** 207–223.

56. Dal Santo, P., Logan, M. A., Chisholm, A. D., and Jorgensen, E. M. (1999) The inositol trisphosphate receptor regulates a 50-second behavioral rhythm in *C. elegans*. *Cell* **98,** 757–767.

57. Carvelli, L., McDonald, P. W., Blakely, R. D., and Defelice, L. J. (2004) Dopamine transporters depolarize neurons by a channel mechanism. *Proc. Natl. Acad. Sci. USA* **101,** 16,046–16,051.

58. Park, K. H., Hernandez, L., Cai, S. Q., Wang, Y., and Sesti, F. (2005) A family of K^+ channel ancillary subunits regulate taste sensitivity in *C. elegans*. *J. Biol. Chem.* **280,** 21,893–21,899.

59. Bianchi, L., Kwok, S. M., Driscoll, M., and Sesti, F. (2003) A potassium channel-MiRP complex controls neurosensory function in *Caenorhabditis elegans*. *J. Biol. Chem.* **278,** 12,415–12,424.

60. Okkema, P. G., Harrison, S. W., Plunger, V., Aryana, A., and Fire, A. (1993) Sequence requirements for myosin gene expression and regulation in *Caenorhabditis elegans*. *Genetics* **135,** 385–404.

61. Brockie, P. J., Madsen, D. M., Zheng, Y., Mellem, J., and Maricq, A. V. (2001) Differential expression of glutamate receptor subunits in the nervous system of *Caenorhabditis elegans* and their regulation by the homeodomain protein UNC-42. *J. Neurosci.* **21,** 1510–1522.

62. Miller, D. M., 3rd and Niemeyer, C. J. (1995) Expression of the *unc-4* homeoprotein in *Caenorhabditis elegans* motor neurons specifies presynaptic input. *Development* **121,** 2877–2886.

14

Sperm and Oocyte Isolation Methods for Biochemical and Proteomic Analysis

Michael A. Miller

Summary

The *Caenorhabditis elegans* gonad is a simple model to investigate molecular mechanisms that regulate fundamental cell and developmental processes. The strength of the model is that *C. elegans* is amenable to genetic manipulation. The complete genome sequence, advances in mass spectrometry, and RNA-mediated interference (RNAi) are now providing a technical infrastructure that complements biochemical and proteomic approaches. This chapter describes simple methods for sperm and oocyte isolation and gonad liberation that can be performed routinely in the lab, without expensive equipment. These methods are ideal for biochemical and proteomic applications, including those aiming to identify proteins based on affinity or biological activity. Germline mRNA expression profiles, RNAi feeding clones, and Gateway®-engineered ORFeome vectors are available to help validate experimental results.

Key Words: Oocyte; sperm; biochemistry; proteomics; mass spectrometry; gonad; reproduction; germ line.

1. Introduction

Caenorhabditis elegans' success as a model organism is primarily owing to the powerful genetic methods with which to study gene function. Biochemical approaches have been impeded by the large amount of starting material required for protein analysis. In the postgenomics era, a collision between two unrelated fields, mass spectrometry and bioinformatics, has changed the rules of protein identification *(1)*. In organisms with sequenced genomes, as little as 10 ng of protein can be identified from one- and two-dimensional polyacrylamide gels *(2)*. Computer algorithms make accurate predictions from isolated protein spots using mass spectra and genome sequence data. Already, *C. elegans* researchers have exploited this technology in biochemical purifications and more general proteomic applications *(3–5)*.

The gonad has several advantages that facilitate proteomic analyses. Germ cells are by far the most abundant cell type in adults and their proteins can be metabolically labeled for quantitative analysis *(5)*. Temperature-sensitive mutants are available that allow comparison of protein content in gonads undergoing spermatogenesis or oogenesis to those lacking germ cells. Transgenes encoding epitope or affinity-tagged proteins can be expressed specifically in the hermaphrodite germline *(6)*, making techniques such as tandem immuno-affinity purification possible. After candidates are identified by mass spectrometry, methods are necessary to validate the results. In this context, genome-wide surveys of genes transcribed during spermatogenesis and oogenesis are available for cross-referencing *(7,8)*. RNA-mediated interference (RNAi) clone libraries facilitate rapid testing of candidate gene function by the feeding method *(9)*, which creates effective knockdowns in oocytes, although not sperm. Finally, an ORFeome library engineered for use with the Gateway® recombination system expedites cloning into yeast two-hybrid vectors to test for direct interactions *(10,11)*.

This chapter describes sperm and oocyte isolation procedures designed for routine practice in the lab. The procedures are biased toward applications requiring less than 2 mL of gametes, such as those aiming to identify proteins from polyacrylamide gels. For biochemical applications that require more material, we provide references for scaling up the procedures. Isolated gametes can be used to purify multiprotein complexes, signaling proteins, or even organelles. Also included is a simple procedure to liberate gonads *en masse* from the tough cuticle, making them readily accessible to extraction buffers.

2. Materials

1. *fog-2(q71)*, *fem-1(hc17)*, *glp-4(bn2)*, or *fer-1(hc1)* mutants. These strains can be obtained from the *Caenorhabditis* Genetics Center (http://biosci.umn.edu/CGC/CGChomepage.htm).

2. LB liquid medium (2–6 L): add 10 g Bacto tryptone, 5.0 g Bacto yeast extract, and 5.0 g NaCl to 1.0 L H_2O, and autoclave (*see* **Note 1**).

3. Alkaline hypochlorite solution (100 mL): prepare fresh by adding 5.0 mL 5 *N* NaOH to 71 mL H_2O and 24 mL bleach.

4. S-medium (1 L): add 5.9 g NaCl to 933 mL distilled deionized (dd) H_2O. Add 50 mL 1 *M* KH_2PO_4 (pH 6.0) and autoclave. When cool, add 3.0 mL 1 *M* $CaCl_2$, 3.0 mL 1 *M* $MgSO_4$, 10 mL trace metal solution (0.364 g $FeSO_4 \cdot 7H_2O$, 0.93 g Na_2EDTA, 0.098 g $MnCl_2 \cdot 4H_2O$, 0.144 g $ZnSO_4 \cdot 7H_2O$, 0.012 g $CuSO_4 \cdot 5H_2O$ in 500 mL ddH_2O), and 1.0 mL cholesterol (5 mg/mL). Store at 4°C and warm to room temperature before use.

5. M9 buffer (1 L): add 3.0 g KH_2PO_4, 6.0 g Na_2HPO_4, and 5.0 g NaCl to ddH_2O, adjust to 999 mL, and autoclave. When cool, add 1.0 mL sterile 1 *M* $MgSO_4$.

6. Egg salts buffer (1 L): add 6.9 g NaCl and 3.58 g KCl to 800 mL ddH_2O. Add 5 mL 1 *M* HEPES, pH 7.4, 2.0 mL 1 *M* $MgCl_2$, and 2.0 mL 1 *M* $CaCl_2$. Bring to 1.0 L with ddH_2O, adjust pH if necessary, and autoclave.

7. X-large Petri dishes (150 mm diameter × 15 mm height).
8. Nitex nylon nets (10, 20, 35, and 45 μm). Lab Paks® containing 12-in. squares are available at Sefar (www.sefar.us).
9. 5-in. Plastic embroidery hoops.
10. 6-in. Benchtop vise (25,000 psi or higher casting) mounted to a 2- × 10- × 40-in. wood slab or lab bench (*see* **Note 2**).
11. 6–8 in. Square Plexiglas plates (two).
12. 6–8 in. Square 0.5 in. thick hard wood spacers (two).
13. Razor blades.
14. Nutator® single-speed orbital mixer or general platform rocker.
15. 15- and 50-mL polypropylene conical centrifuge tubes.
16. Glass Pasteur pipets with bulbs.
17. Automatic pipetor with 10-mL disposable pipets.
18. Liquid nitrogen and Dewar flask for quick freezing.

3. Methods

3.1. Sperm Isolation

This procedure is modified from that of Klass and Hirsh *(12)*. It relies on nylon nets with precise pore sizes for filtration, and pressure to liberate sperm from males. We use *fog-2(q71)* feminizing mutations to generate populations consisting of an equal ratio of males and females *(13)*. Males are separated from females using a 35-μm net. Sperm preps are often greater than 90% pure with a single pass of filtration, and most yields are between 100 and 200 μL sperm. Because the isolation steps can be accomplished in 1 d, multiple procedures can be performed in 1 wk if *fog-2(q71)* cultures are staged. As much as 2 mL of sperm can be isolated in 1 mo.

1. Grow *fog-2(q71)* animals on 4 X-large nematode growth medium (NGM) plates, ensuring that the animals are well fed (*see* **Note 1**).
2. When gravid adults are abundant, collect the worms by pipetting 10 mL of M9 buffer onto a plate. Tilt the plate back and forth, then dump worms and buffer onto the next plate. Continue until all worms are floating in a single plate. Transfer worms with a glass Pasteur pipet to a 15-mL conical polypropylene centrifuge tube. To collect the residual worms from the plates, repeat this process with 10 mL fresh M9 buffer. Centrifuge both tubes in a clinical centrifuge for 2 min at 600*g*. Remove M9 buffer and combine worms into a single polypropylene tube. Centrifuge again and remove as much buffer as possible.
3. Add 5 mL freshly made alkaline hypochlorite solution to worms. Cap and vortex for 3–4 min, then centrifuge at 800*g* for 45 s (*see* **Note 3**). Hatched worms are dissolved, but eggs survive. Quickly wash the eggs with 5 mL M9 buffer, repeat four more times, then resuspend them in S-medium. Incubate with rocking for approx 20 h at 25°C. The following day, estimate the number of hatched L1 larva per μL S-medium.
4. Plate L1 larva onto 40–60 X-large NGM plates at a density of 6000–10,000 per plate. Grow for approx 60 h at 25°C until the plates contain males and gravid females (*see* **Note 4**). Add *Escherichia coli* concentrate as necessary (*see* **Note 1**).

5. Float animals off the plates with M9 buffer as in **step 2**. Transfer them to a 50-mL polypropylene tube or several 15-mL tubes. Centrifuge at 600g for 2 min. Combine worms to a single 15-mL tube and wash twice with M9 buffer to remove bacteria.

6. Gravid females have a diameter greater than 40 μm and cannot swim through a 35-μm net; males are thinner and can freely pass through. Place a 35-μm net in an embroidery hoop. Next, place the hoop in an X-large Petri dish so that the net faces the bottom. Place two small spacers, such as stacks of glass slides, underneath the perimeter of the hoop so that it is raised approx 0.5 cm off the dish bottom. The hoop will form a barrier around the perimeter of the net. Using a glass pipet, transfer worms to the net. Add M9 buffer to the worms until the buffer level is approx 0.5 cm above the net. Sift animals back and forth every few minutes. After 15–20 min, use a stereo microscope to examine male accumulation in the plate. Wait until most males have passed through the net. Collect the males and spin at 600g for 2 min. Remove the supernatant, leaving one additional volume M9 buffer above the volume of males.

7. Carefully applied pressure to males will trigger release of sperm. Place concentrated males on a Plexiglas plate. Make sure that the worms are positioned so they are near the contact site of the vise arms, where the pressure is highest. Sandwich males between the two Plexiglas plates, and then sandwich the Plexiglas plates between the wood spacers. Place the plates in the vise so the worms are directly between the arms, and apply pressure. To determine whether sperm are being liberated, remove the Plexiglas plates from the vise and examine males using a stereo microscope. Sperm appear as small dots that refract light (*see* **Note 2**).

8. When most males have released their sperm, separate the plates. Quickly add M9 buffer to the carcasses to prevent sperm dehydration. Wash off sperm and carcasses into an X-large Petri dish. Use a razor blade if necessary to sweep sperm off the plates. Repeat **steps 7** and **8** until all males have been used.

9. Sperm have a diameter of approx 5 μm and can freely pass through a 10-μm net. Place a 10-μm net in an embroidery hoop and place the hoop in an X-large dish with the net face down, as in **step 6**. Using a Pasteur pipet, transfer sperm and carcasses to the net so there is an even distribution. Slowly add M9 buffer and wash. Once the buffer level reaches 0.5 cm above the net, shake the net back and forth, and up and down (*see* **Note 5**). Sperm will accumulate in the bottom of the dish.

10. Transfer sperm to a 15-mL polypropylene tube(s) and centrifuge at 800g for 10 min. Remove all but 1 mL of M9 buffer and the sperm pellet. Resuspend the pellet and take a 2-μL aliquot to check for purity under a stereo or high power microscope. If the purity is not satisfactory, add 10 mL fresh M9 and centrifuge at 600g for approx 5 min, until about 95% of sperm are in the pellet. Remove the supernatent, which contains less dense debris, and repeat. Alternatively, perform a second filtration step with the 10-μm net.

11. When sperm purity is satisfactory, spin the sperm again and transfer them to an Eppendorf tube (*see* **Note 6**).

12. Combine the preps and spin sperm in a microcentrifuge at high speed. Remove all M9 buffer from the sperm pellet, and freeze it quickly in liquid nitrogen. Store at –80°C.

3.2. Oocyte Isolation

In addition to sperm, oocytes can be isolated using nylon nets *(14,15)*. One of two temperature-sensitive mutant strains is used as starting material. *fer-1(hc1)* hermaphrodites produce defective sperm at 25°C *(16)*. The mutant sperm still stimulate oocyte meiotic maturation and ovulation, so large numbers of unfertilized oocytes can be isolated. However, most of these oocytes are endomitotic and exhibit features typical of necrotic cells. *fem-1(hc17)* hermaphrodites produce no sperm whatsoever at 25°C *(17)*, and many isolated oocytes have not been ovulated. The disadvantage with this strain is that significantly fewer oocytes are isolated. In most cases, the yield using *fer-1(hc1)* is between 400 and 800 μL of oocytes.

1. Grow *fer-1(hc1)* or *fem-1(hc17)* animals on four X-large NGM plates at 15°C, ensuring that animals are well fed (*see* **Note 1**). When the plate is full of gravid adults, perform alkaline hypochlorite treatment to generate a synchronized L1 stage population, as in **Subheading 3.1.**, **steps 1–3**. Remember to incubate eggs overnight at the restrictive temperature, 25°C (*see* **Note 3**).
2. Plate L1 larva onto 40–60 X-large NGM plates at a density of 6000–10,000 per plate. Grow at 25°C until adult worms are full of oocytes, about 65–75 h after plating. Add *E. coli* concentrate as necessary (*see* **Note 1**).
3. Collect worms in egg salts buffer by floating them off the plates, as in **Subheading 3.1.**, **step 2**. Transfer them to a 50-mL polypropylene tube or several 15-mL tubes. Centrifuge at 600*g* for 2 min to pellet the worms. Combine tubes, if necessary, and wash twice with egg salts buffer to remove bacteria (*see* **Note 7**).
4. Transfer animals to a 60- × 15-mm Petri dish. Fill the dish so it is about half full with densely packed worms. When females are cut in egg salts buffer, their gonads tend to extrude into the surrounding medium. Continued cutting releases oocytes from the reproductive tract. With a clean razor blade, cut worms for 5 min using rapid up and down motion. Check under a stereo microscope for extruded gonads and free oocytes. Continue until nearly all worms have been cut. Massive numbers of oocytes should be observed in the dish (*see* **Note 8**).
5. Most oocytes are between 25 and 40 μm in diameter, and will pass freely through a 45-μm net. Place an embroidery hoop containing a 45-μm net in an X-large Petri dish, so that the net faces the bottom of the dish, as in **Subheading 3.1.**, **step 6**. Using a glass pipet, transfer the oocytes and carcasses to the net so they are evenly distributed. Add egg salts buffer to the top of the net and wash. Once the buffer level reaches 0.5 cm above the net, sift the carcasses back and forth, and up and down. Check using a stereo microscope to determine whether oocytes are passing through the net.

6. Transfer the oocytes from the bottom of the dish to two 15-mL polypropylene tubes and spin at 600*g* for 4 min. Remove all but 4-mL buffer from each tube and resuspend the oocytes. Transfer the suspension to a new X-large Petri dish containing a 20-μm net, and wash as in the previous step. Smaller contaminating particles will pass through the net, but oocytes will not. Collect oocytes into a new Petri dish by washing the net with egg salts buffer. Transfer oocytes to two 15-mL disposable conical tubes, and spin at 600*g* for 5 min to pellet the oocytes. Remove the buffer leaving one additional volume of buffer above the volume of oocytes.

7. Gently resuspend the oocytes, take a 2-μL aliquot, and examine the purity using a stereo or high power microscope. Generally, a single pass of filtration yields approx 60–80% pure oocytes. Excess debris can be removed from oocytes by low speed centrifugation or repeated filtration. If more worms remain from **step 3**, repeat the procedure. Combine the preps, and spin to pellet oocytes. Remove the supernatant and freeze oocytes quickly in liquid nitrogen. Store at –80°C.

3.3. Gonad Liberation

For some applications, isolated gametes are not required. Complete extraction from whole worms is relatively straightforward, but it results in a complex mixture of total protein. Liberating gonads into the surrounding medium makes them readily accessible to extraction buffers, including those that are likely to maintain protein complexes. With the exception of intestinal and gonadal cells, other cell types tend to remain in the body column, where the tough cuticle helps protect them from extraction. Temperature-sensitive mutant strains can be used to evaluate germ line specificity. For example, to test whether a given protein is expressed in the germ line, *fem-1(hc17)* mutants, whose germ line is feminized *(17)*, are compared with *glp-4(bn2)* mutants, which lack germ cells *(18)*. To test whether a protein is expressed in germ cells committed to the oocyte fate, *fem-1(hc17)* mutants are compared with *fem-3(q20)* mutants, whose germ line is masculinized *(19)*. These strains have the additional advantage that they do not produce progeny at the restrictive temperature, leaving a homogeneous population of adults for extraction.

1. Grow *fem-1(hc17), fem-3(q20)*, or *glp-4(bn2)* mutants on four X-large NGM plates at 15°C, ensuring that animals are well fed (*see* **Note 1**). When gravid hermaphrodites are abundant, perform alkaline hypochlorite treatment, as described in **Subheading 3.1.**, **steps 1–3**. Incubate eggs overnight at 25°C (*see* **Note 3**).

2. Plate L1 larva onto 40–60 X-large NGM plates at a density of 6000–10,000 per plate. Grow at 25°C for approx 60 h, adding concentrated *E. coli* as necessary (*see* **Note 1**).

3. Collect worms as described in **Subheading 3.1.**, **step 2**, except using egg buffer instead of M9 buffer. Transfer them to a single 50-mL polypropylene tube or several 15-mL polypropylene tubes, and centrifuge at 600*g* for 2 min. Transfer worms to a single 15-mL tube, and wash twice with egg salts buffer to remove

bacteria. Using the procedure described in **Subheading 3.2.**, **step 4**, liberate gonads with a razor blade, and monitor progress using a stereo microscope. Continue until nearly all worms have been dissected and gonads are exposed in the medium. Using a glass pipet, transfer the gonads and carcasses to a 15-mL polypropylene tube, and centrifuge in a clinical centrifuge at 800*g* for 5 min. Remove the supernatant, and freeze the worms quickly in liquid nitrogen. Store at –80°C.

4. Notes

1. Food is required for gamete proliferation and growth. If worms consume all bacteria on the plates, additional food must be added. Grow 2.0 L OP50 or NA22 bacteria in LB medium overnight at 37°C until the culture is dense. Centrifuge for 20 min at 1500*g* to pellet the bacteria. Pour off the supernatant and resuspend bacteria in 40 mL M9 buffer. Store the *E. coli* concentrate at 4°C. Add food to worms as needed, and allow plates to dry. As an alternative, use chicken eggs to grow a rich food source *(12,15)*.

2. We mounted a 42-lb vise onto a 2- × 10- × 40-in. piece of lumber, creating a portable worm smasher that can be placed next to a stereo microscope. Alternatively, the vise can be mounted to a lab bench. Worms should be oriented on the Plexiglas plate so they receive maximum pressure. A good tug on the vise handle is required to trigger sperm liberation (these guys are tough). Higher worm densities require greater pressure. The wood spacers help protect the Plexiglas plates from being damaged, but they are not necessary. More pressure can be generated without them. For preps containing more than a million males, a laboratory press is recommended *(12)*.

3. To ensure that eggs remain viable, but carcasses are dissolved, vortex worms in alkaline hypochlorite solution for 3 min, then spin in a clinical centrifuge. Worm carcasses are dark brown, whereas eggs are much lighter. If greater than 20% of the pellet is dark brown, remove the old alkaline hypochlorite solution and add 5 mL fresh solution. Vortex for 30 s to 2 min, depending on the fraction of carcasses that remain, then centrifuge again and wash the eggs with M9 buffer. If the alkaline hypochlorite treatment is too long or the eggs are not washed thoroughly, many will not hatch.

4. When growing worms on X-large NGM plates, avoid overcrowded conditions. Crowding can trigger dauer formation and loss of synchrony in growth. Good separation of males from females requires that the population consist almost entirely of adults. The optimal time for separation is when nearly all females are gravid, yet few progeny have hatched on the plates. Larvae can be problematic because they pass through 35-μm nets with the males. If your culture has too many larvae, they can be separated from adults using a 20-μm net.

5. Worm carcasses get trapped in the net and prevent sperm and oocyte flow. If the net gets too crowded, switch to a clean net to achieve maximum yields.

6. Sperm isolated from males have not been activated, and are not motile. Activation can be stimulated in vitro using Pronase or triethanolamine in sperm medium *(20)*.

7. If small larvae are found in the *fer-1(hc1)* cultures, separate them from adults using a 20-µm net. *See* **Subheading 3.1.**, **step 6** for the separation technique.

8. Cutting worms with a razor blade releases oocytes from the proximal gonad and uterus. Alternative methods using chemicals or sonication release oocytes from the uterus *(14,15)*. Although these methods have the advantage that less contaminating debris is generated, the liberated oocytes are endomitotic and exhibit features typical of necrotic cells. Others have used a small blender to release oocytes.

References

1. Johnson, R. S., Davis, M. T., Taylor, J. A., and Patterson, S. D. (2005) Informatics for protein identification by mass spectrometry. *Methods* **35,** 223–236.

2. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem.* **68,** 850–858.

3. Miller, M. A., Nguyen, V. Q., Lee, M. H., et al. (2001) A sperm cytoskeletal protein that signals oocyte meiotic maturation and ovulation. *Science* **291,** 2144–2177.

4. Cheeseman, I. M., Niessen, S., Anderson, S., et al. (2004) A conserved protein network controls assembly of the outer kinetochore and its ability to sustain tension. *Genes Dev.* **18,** 2255–2268.

5. Krijgsveld, J., Ketting, R. F., Mahmoudi, T., et al. (2003) Metabolic labeling of *C. elegans* and *D. melanogaster* for quantitative proteomics. *Nat. Biotechnol.* **21,** 927–931.

6. Praitis, V., Casey, E., Collar, D., and Austin, J. (2001) Creation of low-copy integrated transgenic lines in *Caenorhabditis elegans*. *Genetics* **157,** 1217–1226.

7. Reinke, V., Smith, H. E., Nance, J., et al. (2000) A global profile of germline gene expression in *C. elegans*. *Mol. Cell.* **6,** 605–616.

8. Reinke, V., Gil, I. S., Ward, S., and Kazmer, K. (2004) Genome-wide germline-enriched and sex-biased expression profiles in *Caenorhabditis elegans*. *Development* **131,** 311–323.

9. Kamath, R. S., Fraser, A. G., Dong, Y., et al. (2003) Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* **421,** 231–237.

10. Li, S., Armstrong, C. M., Bertin, N., Ge, H., Milstein, S., Boxem, M., Vidalain, P. O., Han, J. D., Chesneau, A., et al. (2004) A map of the interactome network of the metazoan *C. elegans*. *Science* **303,** 540–543.

11. Reboul, J., Vaglio, P., Rual, J. F., et al. (2003) *C. elegans* ORFeome version 1.1: experimental verification of the genome annotation and resource for proteome-scale protein expression. *Nat. Genet.* **34,** 35–41.

12. Klass, M. R. and Hirsh, D. (1981) Sperm isolation and biochemical analysis of the major sperm protein from *C. elegans*. *Dev. Biol.* **84,** 299–312.

13. Schedl, T. and Kimble, J. (1988) *fog-2,* a germ-line-specific sex determination gene required for hermaphrodite spermatogenesis in *Caenorhabditis elegans*. *Genetics* **119,** 43–61.

14. Stroeher, V. L., Kennedy, B. P., Millen, K. J., et al. (1994) DNA-protein interactions in the *Caenorhabditis elegans* embryo: oocyte and embryonic factors that bind to the promoter of the gut-specific *ges-1* gene. *Dev. Biol.* **163,** 367–380.
15. Aroian, R. V., Field, C., Pruliere, G., Kenyon, C., and Alberts, B. M. (1997) Isolation of actin-associated proteins from *Caenorhabditis elegans* oocytes and their localization in the early embryo. *Embo. J.* **16,** 1541–1549.
16. Ward, S. and Miwa, J. (1978) Characterization of temperature-sensitive, fertilization-defective mutants of the nematode *Caenorhabditis elegans*. *Genetics* **88,** 285–303.
17. Doniach, T. and Hodgkin, J. (1984) A sex-determining gene, *fem-1*, required for both male and hermaphrodite development in *Caenorhabditis elegans*. *Dev. Biol.* **106,** 223–235.
18. Beanan, M. J. and Strome, S. (1992) Characterization of a germ-line proliferation mutation in *C. elegans*. *Development* **116,** 755–766.
19. Barton, M. K., Schedl, T. B., and Kimble, J. (1987) Gain-of-function mutations of *fem-3*, a sex-determination gene in *Caenorhabditis elegans*. *Genetics* **115,** 107–119.
20. Ward, S., Hogan, E., and Nelson, G. A. (1983) The initiation of spermiogenesis in the nematode *Caenorhabditis elegans*. *Dev. Biol.* **98,** 70–79.

15

Preservation of *C. elegans* Tissue Via High-Pressure Freezing and Freeze-Substitution for Ultrastructural Analysis and Immunocytochemistry

Robby M. Weimer

Summary

High-pressure freezing (HPF) is capable of converting liquid water, to a depth of approx 0.6 mm, into amorphous ice nearly instantaneously. At midbody, an adult *Caenorhabditis elegans* hermaphrodite approaches its widest girth of approx 0.1 mm. In theory, an entire living adult animal can be physically immobilized instantly in amorphous ice by HPF, thus, providing a unique opportunity to examine cellular architecture with exquisite spatial preservation. The following chapter will discuss, in detail, procedures for freezing *C. elegans* under high pressure, for embedding frozen samples in resin after a freeze-substitution step, and for the postembedding immunogold labeling of proteins contained within thin sections of embedded animals. These protocols enable high-resolution analysis of both morphological features and molecular domains within most tissues of *C. elegans*.

Key Words: *Caenorhabditis elegans*; immuno-electron microscopy; high-pressure freezing; freeze-substitution; immunocytochemistry; immunogold; postembedding.

1. Introduction

Few animals have been studied as thoroughly as the nematode *Caenorhabditis elegans* by electron microscopy. Take, for example, the *tour de force* of White et al. *(1)* in which the structure and connectivity of the adult hermaphrodite nervous system was delineated by reconstruction of serial electron micrographs. Similar efforts have described the anatomy of *C. elegans* in detail *(2–5)* and have proven invaluable in the study of its biology. However, the nematode represents a challenge for electron microscopists: its tissues are difficult to preserve for ultrastructural studies, especially when one tries to also preserve protein antigenicity for immunocytochemistry. This is owing primarily to the animal's low-

permeable cuticle, which slows the diffusion of solutions into the animal during chemical fixation and embedding. This chapter details a relatively recent technical advance in the fixation of *C. elegans* for ultrastructural studies, which helps circumvent this problem: the use of high-pressure freezing (HPF) to physically immobilize living animals rapidly (within milliseconds) in vitreous ice *(6–19)*. This enables chemical fixation, dehydration, and embedding to occur at low temperatures over an extended period of time without morphological deterioration.

The cryofixation of biological samples by HPF relies on the following three physical phenomena associated with water (for a detailed discussion *see* **ref. *20***):

1. The rapid cooling of liquid water can solidify it as vitreous ice *(21,22)*, which is a noncrystalline, or glass-like, form of ice that does not undergo expansion during solidification.
2. Increasing the pressure exerted on water decreases the temperature at which homogenous ice crystal nucleation occurs.
3. As pressure increases so does the viscosity of water, which, in turn, decreases the growth of ice crystals.

Under normal atmospheric conditions water must be cooled at a rate greater than 10,000°C/s for vitrification to occur—a rate that can only be reached at superficial surfaces of tissues (~20 μm deep). However, under high-pressure (~2100 bar), homogenous ice crystal nucleation and ice crystal growth are significantly suppressed, therefore, decreasing the cooling rate required for vitrification, and, as a consequence, increasing the depth at which biological samples can be vitrified by rapid freezing (to a depth of ~0.6 mm). Fortuitously, an adult *C. elegans* hermaphrodite is roughly 0.1 mm in diameter at midbody, thus, in theory a living animal can be instantly immobilized by HPF and its ultrastructural features preserved in vitreous ice.

Once vitrified, frozen samples can either be stored for long durations under liquid nitrogen or processed directly and studied in several ways: direct viewing on a microscope cryostage, cryosectioning followed by cryoelectron microscopy *(23)*, freeze fracture *(24)*, or freeze substitution, to name a few. The latter affords both the ease of utilizing conventional laboratory equipment (microtomes, grid stage, and others) for examining samples with the electron microscope and is compatible with postembedding immunolabeling of cellular proteins.

During freeze substitution, frozen cellular water is replaced by organic solvent molecules (typically acetone or methanol) at low temperatures between –78 and –90°C (for a more in-depth review *see* **refs. *25*** and ***26***). This allows for the dehydration of vitrified samples without raising the temperature above –70°C—a temperature at which secondary ice crystals begin to form in biological samples (for discussion regarding cubic ice formation at low temperatures *see* **ref. *26***). Concurrently, chemical fixatives and contrasting agents can

be added to the substitution solvent to help stabilize cellular structures and add contrast. However, the exact chemistry of low-temperature chemical fixation and staining remains unknown, therefore, a researcher may need to determine, empirically, a protocol suitable for their specific experimental needs. This chapter outlines two different freeze-substitution protocols: one which results in well-contrasted membranes and is useful for detailed morphmetric analysis, whereas the other is capable of preserving immunoreactivity as well as ultrastructure and is useful for high-resolution immunocytochemical studies by postembedding immunogold labeling.

Postembedding immunocytochemistry takes advantage of the fact that most proteins retain antigenicity after weak fixation and embedding in an appropriate resin (for a complete review *see* **ref. 27**). A typical postembedding immunogold labeling experiment consists of incubating thin section with a primary antibody, during which time the antibody binds to those antigens that are exposed at the surface of the section, followed by an incubation with a gold-conjugated secondary antibody to reveal the location of the primary antibody under the electron beam. Because only those proteins at the surface of the section will be stained, the disadvantage of postembedding is that staining intensity is usually low. However, the advantages of postembedding immunogold staining are threefold. First, postembedding staining does not require samples to be permeablized before fixation, a process that typically introduces structural artifacts. Second, if a tissue of interest is present in multiple sections, several antibodies can be used per fixed sample. Third, if antibodies from different species are used, sections can be double labeled when revealed with different sized gold bead secondary antibody conjugates. An additional advantage specific to staining tissues after cryofixation is that protein antigenicity, as well as structure, tends to be preserved better than with standard room-temperature fixation.

Several factors should be considered before using HPF and freeze substitution for the fixation of *C. elegans*. First, the cost: high-pressure freezers are expensive (~$150,000), making their availability, at the moment, limited to a small number of research institutes. Second, time: HPF is quick; however, freeze substitution takes longer than standard fixation techniques. Third, the potential difficulty in the staining/revealing of your structure of interest: as previously mentioned, the chemistry of chemical fixation and staining at low temperatures is still an open-ended question. Therefore, time may need to be invested to optimize the staining of a specific structure of interest. However, the quality of preservation afforded by HPF is currently second to none. Cellular structures can be preserved in their native state by ultrarapid vitrification, whereas protein antigenicity can be preserved by low-temperature dehydration and embedding. Both of which enables the high-resolution study of morphology and molecular domains within *C. elegans* tissues.
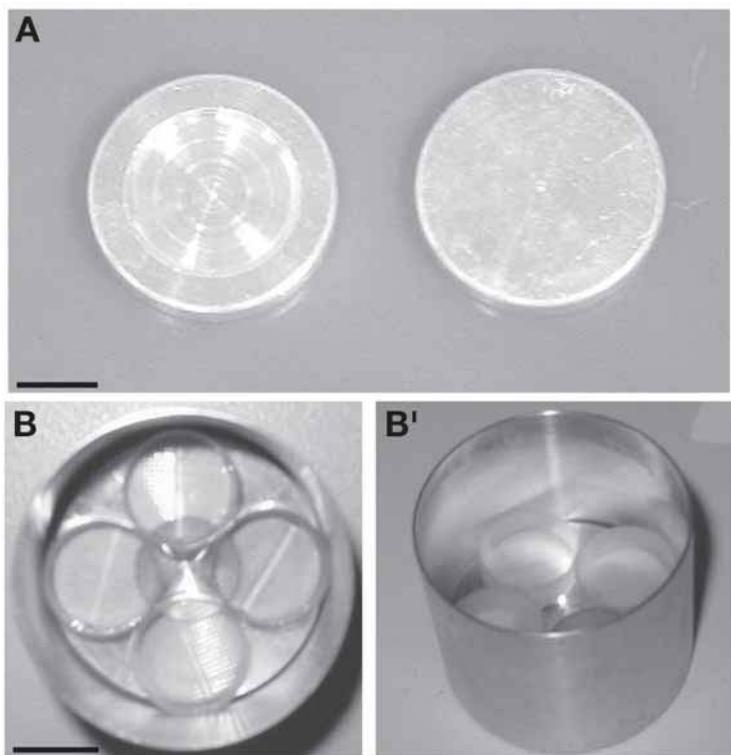
Fig. 1. Freezing and freeze-substitution solution chambers. **(A)** A freeze chamber consists of a 100-μm deep Type A chamber (left) and a flat topped Type B chamber (right). Bar = 1 mm. **(B)** A solution chamber for freeze substitution can be constructed of a universal container, a scoring wheel, and four plastic mesh bottom vials. **B** and **B'** are top and oblique views, respectively, of one such chamber. Bar = 10 mm.

2. Materials

2.1. High-Pressure Freezing

1. High-pressure freezer: protocols in this chapter have been performed with the BAL-TEC HPM 010 (BAL-TEC, Liechtenstein) and accompanying accessories (specimen holder and unloading device; *see* **Note 1**).
2. Freeze chambers are each composed of one Type A (BAL-TEC, cat. no. LZ 02137 VN) and one Type B (BAL-TEC, cat. no. LZ 02138 VN) aluminum specimen carrier (**Fig. 1A**). When assembled the sample will be contained within a cylindrical space of 2 mm in diameter and 0.1 mm in height. Similar specimen carriers are available from other manufacturers.
3. Liquid nitrogen.

4. Insulated tweezers with a long reach (Leica, Austria, cat. no. 701955; or any other supplier).
5. Bacteria (*Escherichia coli* strain OP50) grown for 1 wk on a worm media plate *(28)*.
6. Animals to be frozen.

2.2. Freeze Substitution and Embedding

2.2.1. Fixation for Morphometric Analysis

1. Freeze-substitution apparatus. Although a homemade freeze-substitution apparatus can be assembled from common lab equipment (*see* **Note 2**), this chapter will detail the utilization of the Leica EM automatic freeze-substitution (AFS) system (Leica) and accompanying accessories (universal containers, scoring wheels, and covers).
2. Plastic mesh bottom vials: the 13- × 4-mm mesh lids to the 13- × 18-mm mesh vials (cat. no. C211) supplied by TAAB (United Kingdom).
   Note: At the time of publication, this item is not being manufactured. An alternative would be to use a similar vial supplied by Leica (cat. no. 702734) trimmed to a height of approx 5 mm to facilitate access to contents.
3. Pure ($\leq$0.5% H_2O) acetone; any supplier (*see* **Note 3**).
4. Freeze-substitution solution: 0.5% gluteraldehyde (diluted from an EM-grade 10% anhydrous acetone stock solution; any supplier), 0.1% tannic acid (Mallinckrodt; *see* **Note 4**) in pure acetone. Prepare fresh before use. Exercise caution when handling gluteraldehyde, it is recommended to wear protective gear (eye protection, gloves, and lab coat) and handle the reagent under a negative-pressure hood.
5. Stain solution: 2% osmium tetroxide in acetone that is made by dissolving electron microscopy (EM)-grade crystalline osmium tetroxide (any supplier) in the appropriate volume of acetone. Osmium tetroxide is sensitive to light. Stock solutions can be stored at –80°C for several months, discard if the solution darkens in coloration. Extreme caution should be exercised when handling concentrated osmium tetroxide: wear double gloves, eye protection, and lab coat and only handle solutions in a negative pressure hood.
6. Araldite 502 embedding kit (any supplier), these reagents can be stored for long durations at room temperature (~18 mo).
7. Embedding capsules, the cap of a BEEM® capsule (any supplier) will suffice.

2.2.2. Fixation for Immunocytochemistry

1. Freeze-substitution machine (AFS; Leica) and accompanying accessories (universal containers, scoring wheels, covers, flat embedding mold *sans* mold, and UV lamp).
2. Pure ($\leq$0.5% H_2O) acetone (any supplier; *see* **Note 3**).
3. Freeze-substitution solution: 0.1% $KMnO_4$ (any supplier) in acetone, which is made the day of use. Care should be taken to ensure that all of the $KMnO_4$ dissolves, filter through paper if necessary.

4. Lowicryl HM20 embedding resin (Electron Microscopy Sciences, cat. no. 14340). The stock reagents can be stored at room temperature for long durations (~18 mo), but the working solution should be made the day of use strictly following the manufacturer's directions (*see* **Note 5**). Lowicryls may cause eczema in sensitive individuals, always wear appropriate protective gear and work in a negative-pressure hood.
5. Embedding capsules, the caps of BEEM capsules (any supplier) are recommended.

2.3. Postembedding Immunogold Labeling

1. Phosphate buffered saline (PBS), pH 7.4.
2. Aurion blocking solution™: the species used to generate the secondary gold-conjugated antibodies will determine which type of normal serum to use for a blocking solution. For example, if goat anti-rabbit gold-conjugated secondary antibodies are to be used then normal goat serum (Aurion, The Netherlands) should be used as a blocking solution.
3. Primary antibody (*see* **Note 6**) diluted in PBS with 0.5% gelatin (cold water fish; Aurion) and 0.001% Tween-20 (Aurion).
4. Gold-conjugated secondary antibody directed against the primary antibody diluted between 1:50 to 1:200 in PBS with 0.5% gelatin and 0.001% Tween-20.
5. 2% Gluteraldehyde in PBS, which is made by diluting EM-grade 10% aqueous gluteraldehyde (any supplier) in PBS. Again, exercise caution when handling concentrated solutions of gluteraldehyde.
6. Deionized H_2O.

3. Methods
3.1. High-Pressure Freezing

Briefly, animals are placed into freeze chambers containing *E. coli*, which is used as a fill to remove air pockets from the freeze chamber (*see* **Note 7**), then frozen in the high-pressure freezer and quickly moved into liquid nitrogen. To begin, prepare the high-pressure freezer for operation, according to the manufacturer's directions, and the necessary workstations. A typical work environment would include a workstation equipped to load animals into freeze chambers, which requires a dissecting microscope and worm pick, the high-pressure freezer, and an unloading area, which is comprised of an insulated box containing an unloading device and filled with liquid nitrogen—it is critical that the unloading area is located in close proximity to the high-pressure freezer because the frozen sample must be rapidly moved from the freezer into liquid nitrogen.

3.1.1. Load the Animals Into Freeze Chambers

1. Fill the freeze chamber with *E. coli*. With tweezers, invert a type A specimen carrier such that the 100-μm well is facing down. Slowly scrape the carrier along the outer growth ring of a plated 1-wk-old *E. coli* OP50 culture to fill the com-

partment. It is critical that the entire chamber is filled with bacteria. Right the carrier and while supporting it with tweezers on top of a flat surface use a worm pick to remove excess bacteria. The level of the bacteria should be flush with the chamber rim and the rim should be free of bacteria (*see* **Note 8**). From here on it is important to work quickly so that the bacteria and worms do not dehydrate before freezing.

2. Load animals into the freeze chamber. With a worm pick, place several animals at a desired developmental stage (*see* **Note 9**) into the center of the freeze chamber loaded with bacteria.

3. Close the freeze chamber by placing a Type B specimen carrier flat side down on top of the filled Type A carrier. A slight force may need to be applied to the top in order to seal the chamber.

3.1.2. Freeze by HPF

1. Secure the loaded freeze chamber in the specimen holder. With tweezers, move the loaded chamber into the clasp of the holder, close the clasp, and rotate to tighten.

2. Place the holder into the pressure chamber of the high-pressure freezer and secure with the locking pin.

3. Freeze by pressing the "jet" button on the BAL-TEC HPM010 control panel. During a freeze cycle, isopropyl alcohol is introduced into the pressure chamber 0.5 s before the introduction of pressurized liquid nitrogen. This two-step process allows the freeze chamber to pressurize by the compression of isopropyl alcohol about 15 ms before it is rapidly frozen by liquid nitrogen.

3.1.3. Transfer the Frozen Sample
to Liquid Nitrogen and Remove From Holder

1. Rapidly move the specimen holder from the freezer into the unloading station, which is filled with liquid nitrogen. It is critical that the frozen specimen remains below –70°C to prevent the growth of secondary ice crystals. Once the pressure chamber in the high-pressure freezer equilibrates to atmospheric pressure, the locking pin can be removed and the specimen holder removed from the freezer. Typically, the locking pin is removed with one hand while the other moves the holder to the nearby unloading station with a rapid arching movement.

2. Remove the freeze chamber from the holder. First, place the clasp of the holder into the unloading device groove and rotate to loosen. Raise the clasp to above the groove and with insulated tweezers open the holder clasp and remove the freeze chamber, keeping the chamber below the surface of the liquid nitrogen. At this point, frozen samples can either be processed directly for several types of experimentation, including freeze substitution (discussed in **Subheading 3.2.**), or stored in liquid nitrogen (*see* **Note 10**).

3. Repeat **Subheadings 3.1.1.**, **step 1** and **3.1.3.**, **step 2** until the desired number of samples are frozen. A complete cycle requires approx 5 min and between each cycle it is important to dry the specimen holder to prevent the clasp from freezing shut—a hairdryer is useful for this task.

3.2. Freeze Substitution and Embedding

To embed the frozen samples in resin for serial sectioning or postembedding immunostaining, the vitreous water within the sample is exchanged with an organic solvent by freeze substitution. Various staining techniques can be used concurrently to add contrast to cellular structures of interest.

3.2.1. Setup of the AFS Apparatus

Although freeze substitution can be carried out using a number of different apparatus (*see* **Note 3**), for the purpose of brevity, this section will describe the procedure for freeze substitution using a Leica EM AFS system. The Leica EM AFS affords control over a wide range of holding temperatures, duration, and the rates of temperature changes.

1. Fill the liquid nitrogen reservoir according to the manufacturer's instructions.
2. Preprogram the appropriate temperatures, duration, and rates into the control unit following the manufacturer's directions. For fixation in osmium (**Subheading 3.2.2.**) the desired temperature profile can be achieved by programming the Leica EM AFS with the following parameters: T1 = –90°C for 96 h, S1 = 5°C/h, T2 = –25°C for 16 h, S2 = 10°C/h, T3 = 4°C 4 h. To fix tissues for immunocytochemistry studies (**Subheading 3.2.3.**), freeze substitution requires the following regime: T1 = –90°C for 96 h, S1 = 4°C/h, T2 = –45°C for 158 h, S2 = 6°C/h, T3 = 20°C 0 h.
3. Equilibrate the temperature within the thermoregulated AFS chamber to the appropriate starting temperature by starting the entered program followed by pressing the pause button. This will start the program, thus, lower the temperature within the chamber but will pause the timer.
4. Prepare the solution chambers that will be used to incubate the frozen samples during substitution. This can be done in the following manner: within a universal container, place four mesh bottom vials (*see* **Note 11**) on top of a scoring wheel, **Fig. 1B**—the scoring wheel is used as a spacer to promote the draining of liquids through the mesh vials during solutions changes (*see* **Note 12**). Fill the chamber with the appropriate freeze substitution solution (*see* **Subheadings 2.2.1.**, **step 3** and **2.2.2.**, **step 3**) to the top of the vials (~10 mL of solution) and place in the thermoregulated chamber of the AFS. Allow at least 15 min for the temperature to equilibrate.
5. Once the temperature of the AFS and freeze solutions are equilibrated, place the frozen sample(s) into individual mesh bottom vials within the filled solution chamber. Again, it is important to keep the sample cold at all times, this transfer can be achieved in the following manner: place the frozen sample, still within the freeze chamber, into a free universal container filled with liquid nitrogen, move the universal container into the AFS chamber (*see* **Note 13**) and with insulated tweezers quickly move the frozen sample from the liquid nitrogen into an individual mesh vial (*see* **Note 14**). Typically, when the frozen sample is moved into the substitution solution, the freeze chamber opens; that is, the Type A chamber

and Type B lid slide apart. If this does not occur use tweezers to open the freeze chamber to facilitate solution exchange.
6. Unpause the AFS timer to initiate the programmed temperature incubations.

3.2.2. Freeze Substitution and Embedding for Morphometric Analysis

The following protocol utilizes a combination of tannic acid and osmium to contrast membranes, and the resin Araldite for embedding. Well-contrasted membranes are advantageous for morphometric analysis, whereas Araldite facilitates the generation of serial sections (*see* **Note 15**).

1. Incubate the frozen samples at –90°C for 4 d in freeze-substitution solution: acetone with 0.5% gluteraldehyde and 0.1% tannic acid. Gluteraldehyde acts as a crosslinking agent, whereas tannic acid acts as a mordant and increases the subsequent staining of membranes.
2. Remove the freeze substitution solution and wash several times over 6 h with –90°C acetone (*see* **Note 12**).
3. Remove the last acetone wash and add the staining solution: 2% osmium tetroxide in acetone—osmium is an electron-dense fixative that crosslinks, primarily, to lipids. Initiate the 5°C/h temperature rise to –25°C, hold for 16 h before raising the temperature to 4°C over 3 h.
4. Wash the sample several times with 4°C acetone while increasing the time interval of incubation over 4 h. The wash has to be started as soon as the specimens reach 4°C because osmium is much more reactive at this temperature than below 0°C. Begin with two 15-min incubations, and then increase to 30 min for a wash, then every hour. After the last solution exchange, remove the solution chambers from the AFS and work at room temperature.
5. Embed the samples in resin (*see* **Note 16**). To do so, remove the now fixed animals from the freeze chamber using suction from a transfer pipet or by scraping with a fine-needle tip (*see* **Note 17**). Infiltrate by incubating the sample in 30% araldite in acetone for 4 h, 70% araldite in acetone for 5 h followed by an overnight incubation in 90% Araldite in acetone at 4°C. The following day incubate the sample in 100% araldite for 8 h with three changes before polymerizing at 60°C for 48 h. Owing to the viscosity of the solutions, it is advisable to place the sample in an embedding capsule, such as the top of a BEEM capsule, before adding 90% araldite.
6. Assess the quality of fixation. Once the resin polymerizes, the orientation of the worms within the block can be determined by viewing under a dissecting microscope, allowing one to mount the block in the desired orientation for sectioning at the microtome. Collect thin sections, counter stain with lead citrate and uranyl acetate, and view at the electron microscope. Membranes should be well contrasted and the section of tissue should lack large voids, which are characteristic of ice damage (*see* **Fig. 2** and **Note 18**).

3.2.3. Freeze Substitution and Embedding for Immunocytochemistry

In the following protocol, frozen samples are dehydrated in acetone while contrasted with $KMnO_4$ before embedding in the hydrophilic resin Lowicryl
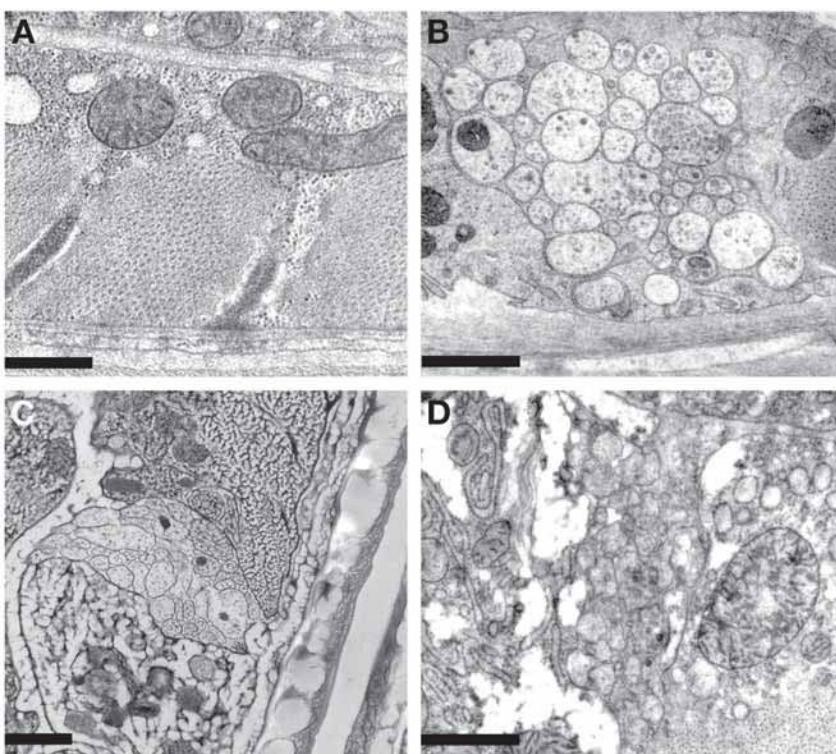
Fig. 2. Examples of well- and not so well-preserved tissues following high-pressure freezing and freeze substitution with tannic acid and osmium. **(A,B)** Micrographs of body-wall muscle and the ventral nerve cord, respectively, of a well-preserved adult hermaphrodite; the section thickness is 40 nm. Notice the well-contrasted plasma membranes, mitochondrial membranes, and synaptic vesicles. Also notice, though, the dark staining of the contractile apparatus in the muscle—structures within the m-line, for example, may be difficult to resolve following this fixation condition (for an example of a well-stained muscle by freeze substitution *see* **ref. *17*** and the conditions therein). In **A**, bar = 500 nm, in **B** it is equal to 1 μm. **(C)** The ventral nerve cord of an animal in which ice crystal damage destroyed most of the ultrastructure. Bar = 1 μm. **(D)** The ventral nerve cord of an animal that was not fully infiltrated with resin before polymerization. Bar = 1 μm. (The micrographs in **B** and **D** are courtesy of Shigeki Watanabe, University of Utah.)

HM20. This fixation is compatible with postembedding immunogold staining cellular proteins (*see* **Note 19**).

1. Incubate the frozen samples at –90°C for 4 d in freeze-substitution solution: acetone with 0.1% KMnO$_4$. Potassium permanganate primarily stains membranes without the severe degradation of immunoreactivity observed with osmium.

2. Raise the temperature within the AFS chamber to –45°C at a rate of 4°C/h.
3. Remove the freeze substitution solution and wash several times with 10- to 30-min intervals until the solution remains clear, approx 2 h (*see* **Note 12**).
4. Remove the sample from the freeze chamber and place at the bottom of the mesh vial. Within the AFS chamber at –45°C, remove the animals and surrounding *E. coli* from the freeze chamber using suction from a transfer pipet or by scraping with a fine needle (*see* **Note 17**). It is desirable for the worm/*E. coli* cake to be removed intact to decrease any mechanical damage to the fixed animal and to decrease the loss of sample. At this point the samples are fragile, exercise caution during the manipulations.
5. Embed the sample in Lowicryl HM20 resin (*see* **Note 5**). Specifically, at –45°C, incubate the sample in 30% Lowicryl in acetone for 12 h, 60% Lowicryl in acetone for 12 h, then three 24-h incubations in pure Lowicryl. Transfer the sample to a BEEM capsule cap resting on a flat embedding mold supplied with the AFS, this acts as a raiser to lift the samples closer to the UV lamp. Fill the BEEM capsule cap with resin then polymerize via UV exposure for 60 h.
6. Raise the temperature within the AFS chamber to room temperature at a rate of 6°C/h.
7. Access the quality of fixation. Once the resin polymerizes, the orientation of the worms within the block can be assessed by viewing under a dissecting microscope, allowing one to mount the block correctly for desired sectioning at the microtome. Collect thin sections, counterstain the sections, and view at the electron microscope (*see* **Fig. 3A** and **Note 20**).

3.3. Postembedding Immunogold Labeling

For postembedding immunocytochemistry, it is preferable to collect thin sections of Lowicryl embedded samples on Formvar-coated nickel slot grids. Nickel grids are inert in solutions used for immunostaining, whereas slot grids provide a relatively large viewing area void of support mesh that can obscure regions of interest. Numerous protocols for postembedding immunocytochemistry have been published, for a complete review *see* **refs. *29*** and ***30***. The following has proven successful for labeling cytoplasmic, structural, and membrane-associated proteins following the previously mentioned fixation protocol.

The following layout can accommodate the staining of 10–15 grids per run. For each wash step, dispense approx 50–100 μL of solution per grid on a Parafilm sheet: to wash 10 grids spot three rows of 10 solution drops on a sheet of Parafilm approx 10×20 cm. It is advisable to filter the wash solutions before use to decrease the accumulation of "dust" particles on the sections. Furthermore, it is beneficial to remove as much solution as possible between changes of solution type, this can be aided by briefly dabbing the edge of the grid on filter paper before placing it in the next solution drop. For incubations of long duration, such as with antibodies, it is advisable to use a humidified chamber to
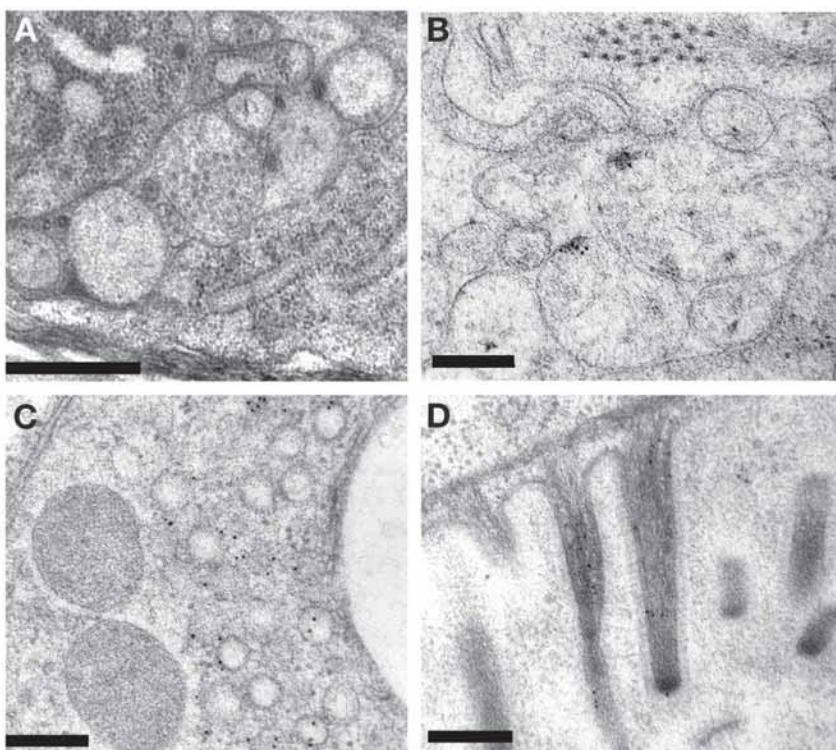
Fig. 3. Immunogold labeling of *Caenorhabditis elegans* tissues fixed with potassium permanganate and embedded in Lowicryl. **(A)** The dorsal nerve cord of an adult hermaphrodite before immunostaining; the section thickness is 50 nm. Bar = 500 nm. **(B)** A 50-nm section stained with a primary antibody directed against SYD-2 *(19)*, a protein required for presynaptic differentiation *(34)*, diluted 1:50, revealed by 10 nm gold-conjugated secondary antibodies diluted 1:50. Notice the decrease in membrane contrast when compared to **A**. This is likely owing to extraction of $KMnO_4$ during immunostaining. Bar = 200 nm. **(C,D)** Examples of immunostaining within the excretory canal and intestine using a primary antibody directed against the B-subunit of the v-type H^+-ATPase *(35)* diluted 1:50 and revealed by 10 nm gold-conjugated secondary antibody diluted 1:50. Bar = 200 nm.

prevent the evaporation of the small volumes of liquid. A humidified chamber can be easily assembled by placing damp paper towels around the periphery of the Parafilm sheet and enclosed by placing an inverted plastic box over both.

1. Incubate the grids in 0.05 *M* glycine in PBS for 15 min (optional; *see* **Note 21**).
2. Move the grids into Aurion blocking solution and incubate for 30 min. This blocking solution decreases nonspecific antibody interactions.

3. Wash three times in PBS; incubate for 5 min each time.
4. Incubate for 1 h in diluted primary antibody.
5. Wash six times in PBS; incubate for 5 min each.
6. Incubate for 1 h in gold-conjugated secondary antibodies diluted 1:50–1:200.
7. Wash nine times in PBS; incubate for 5 min each.
8. Fix the antibody conjugates by incubating in 2% gluteraldehyde in PBS.
9. Wash once in PBS for 5 min.
10. Wash twice in deionized water for 5 min each.
11. Allow the grid to dry before viewing in the electron microscope. It is advisable to counterstain the sections with uranyl acetate and lead citrate before viewing as much of the $KMnO_4$ contrasting agent can be extracted during immunostaining (*see* **Fig. 3B–D** and **Note 22**).

4. Notes

1. At the moment, two commercially available high-pressure freezers are typically used for freezing: the Leica EMPact (Leica) and the BAL-TEC HPM 010 (BAL-TEC). The Leica model offers several benefits: its compact size, its mechanism for handling frozen samples (an automated ejection arm), the ability to use different types of freeze chambers to accommodate various sample types, a graphical readout of freezing conditions as the sample is being frozen, lower liquid nitrogen consumption rates, and its price. However, the author had difficulty obtaining adequately frozen samples using the current version of the Leica EMPact. Therefore, the methods described in this chapter have been performed with the BAL-TEC.
2. Besides Leica, BAL-TEC offers a commercially available automated substitution system, the FS 7500 freeze substitution system, which provides regulated control over temperature and temperature gradients. An alternative substitution system can be assembled from common lab items: a Styrofoam box, an aluminum block, a thermocouple, and dry ice. For further details *see* **ref. *31***.
3. The goal of freeze substitution is to remove water from the frozen sample so that ice crystallization will not cause damage when the sample is warmed; therefore, it is critical to use anhydrous solvents for this step. However, there have been reports of successful substitutions when small amounts of water are present in the solvent *(32)*.
4. Commercial tannic acids are a mixture of polyphenolic compounds and vary considerably between manufactures. For the desired staining of membranes, the correct concentration of tannic acid for each manufacturer or even each lot may need to be determined empirically.
5. Oxygen inhibits the polymerization of Lowicryl. When handling the solution try not to introduce excess air by repeat pipetting or stirring. Bubbling nitrogen gas through the solution can remove excess oxygen.
6. Immunoelectron microscopy is not the technique to use to characterize antibodies; it is best to use well-characterized antibodies. The optimal antibody dilution is, of course, antibody specific. As a starting point, dilute the antibody roughly

10-fold less than you would for *in situ* staining or Western blotting. For double labeling experiments, two antibody sera from two different species can be mixed and used as a primary staining cocktail followed by staining with a cocktail of two different sized gold-conjugated secondary antibodies, one size specific for each primary antibody.

7. Air pockets within the freeze chamber act as insulation, thus, decreases the cooling rate within the sample. In addition, these pockets can collapse under high pressure, causing damage to the freeze chamber and sample. Therefore, it is imperative to fill the freeze chamber completely with material. Because excess water increases the chance of ice damage, it is preferable to use a filling material with relatively low water content and that will not adversely affect the physiology of the animals and introduce artifact. In this protocol, week-old *E. coli* is used as a fill for two reasons: its water content is relatively low and it is a medium that the animals usually encounter when reared in the laboratory. However, other materials have been used as fill, such as, yeast paste *(7)*, agarose *(12)*, and 1-hexadecene *(18)*.

8. Removing excess bacteria from the freeze chamber rim aids in preventing the animals from swimming out of the chamber. When the nose of an animal contacts the metal, the animal typically reverses direction.

9. The number of animals loaded into the freeze chamber can vary depending on the type of experiment. For example, if embryos are of interest, the freeze chamber can accommodate many; for freezing adults, place approx 10–15 animals, just following the L4 to adult molt, into the freeze chamber. When loading the sample, try to be gentle so that artifact is not introduced due to mechanical damage.

10. Frozen samples can be stored for long durations in liquid nitrogen. One way to do this is to place the frozen sample into a Nalgene® cryotube in which a few small holes have been drilled—the holes allow nitrogen gas to escape; therefore, tubes remain submerged. The tubes can be moved into a cryostorage box and stored in a liquid nitrogen storage tank.

11. This particular mesh vial allows access to the frozen sample, which is important when removing the sample from the freeze chamber at low temperatures before embedding. To track the identity of each sample, one can place a notch in the wall of one vial and place it in the 12 o'clock position within the universal container, then note which sample is placed in the 12, 3, 6, and 9 o'clock positions.

12. When changing solutions during freeze substitution, it is important to remove as much liquid as possible without removing the sample. Within the solution chamber, aspirate from the bottom of the universal container which can be accessed through the hole in the scoring wheel spacer. This will draw the solution through the mesh bottom of the plastic vials preventing the loss of samples and remove most of the solution. The next solution can be added by dispensing the solution at the wall of the universal chamber. Do not fill the chamber above the top of the vials because samples can be washed over its walls and lost.

13. When moving a universal container filled with liquid nitrogen, insulated tweezers or pliers can be used to grasp the container. Once within the AFS chamber it is advisable that one hand is used to keep the container elevated, while the other

moves the frozen sample into the freeze-substitution solution using insulated tweezers. Placing the liquid nitrogen-filled universal chamber on the floor of the AFS chamber will result in the boiling of the liquid nitrogen.

14. Do not panic when the acetone freezes as you place the frozen sample into the substitution solution. At –90°C, acetone is near its freezing temperature, the liquid nitrogen-cooled insulated tweezers used to transfer the frozen sample will often cool the acetone solution below its freezing temperature. Two strategies can be taken: (1) move the sample into acetone then retrieve the tweezers quickly before the acetone freezes. This strategy can work, however, you want to make sure that when you are moving your sample into acetone that it becomes submerged, in doing so the tweezers may become frozen in acetone. (2) Work slowly in the acetone to ensure the proper placement/submersion of the frozen sample into acetone, and then use another set of tweezers to scrape the acetone free from the frozen tweezers. This will require one to temporally place the liquid nitrogen-containing universal chamber in or near the AFS to free a hand for manipulating another set of tweezers. In the end, the acetone will quickly return to a liquid state with no detriment to the frozen sample.

15. This freeze-substitution protocol works well for visualizing membranes, in particular, plasma membranes, mitochondrial membranes, and synaptic vesicles, however, may over stain other structures, such as the contractile apparatus. Several other freeze-substitution protocols have been used to study the morphology of *C. elegans* *(7,12,18,31)* and may need to be considered when designing a freeze-substitution experiment.

16. At this point, the animals are encased in fixed *E. coli*. Although one could try to remove the bacteria and change the orientation of the animals, doing so introduces the possibility of damaging the fixed worms by mechanical forces before they are embedded.

17. If a needle is used to pry the sample free, first try scraping the inside wall of the freeze chamber to avoid damaging the animals within the bacteria.

18. Troubleshooting: several problems may arise during freezing and freeze substitution with tannic acid and osmium. These include undesirable dark staining of structures of interest (**Fig. 2A**, muscle m-line), ice crystal damage (**Fig. 2C**), poor resin infiltration (**Fig. 2D**), and poor resin polymerization. The first problem is specific for substitution with tannic acid and osmium: whereas membranes are well contrasted, other structures of interest may be too electron-opaque. As with most fixation techniques for electron microscopy, one condition will not be ideal for every study. To decrease staining, try decreasing the concentration of osmium and/or its incubation time at –25°C, or try staining without the use of tannic acid. Ice crystal damage may be introduced in several ways: improper freezing (consult the manufacturer's guidelines for testing the operation of the high-pressure freezer); the warming of the sample during its transfer to freeze substitution (try to work quickly to minimize the exposure of the sample to warmer environments); and the incomplete removal of water during freeze substitution (make sure the solutions used for substitution are anhydrous and that the

low temperatures are maintained during substitution). Increase the duration of substitution at low temperatures if necessary. Resin infiltration can be problematic. The typical fix is to increase the duration during each step of infiltration. An additional problem can be the poor polymerization of resin, which hinders sectioning. Before diluting make sure to remove excess oxygen from the pure Araldite solution by applying a vacuum as oxygen can inhibit polymerization. If a block is poorly polymerized, try incubating it at 60°C for a longer duration.

19. This freeze-substitution protocol has proven successful for preserving immunoreactivity of many protein types; however, because of the unpredictability of antigen preservation, it may not be ideal for every type of immunolabeling experiment. Other freeze-substitution protocols have been used to study the location of cellular proteins in *C. elegans (11,17)* and should be considered, as well as fixation conditions used for other sample types *(31,33)*, when designing an experiment.

20. Similar to fixation with osmium, one may observe ice crystal damage and poor resin infiltration. If so, *see* **Note 18**. An additional problem specific to Lowicryl resins is its tendency to wick out of the mold, leaving the sample embedded in little or no resin. To prevent this, make sure the wall of the casting mold is not touching another surface and do not over fill the mold, but do not under fill as well. Because of the problematic nature of Lowicryl, it is advisable to try a mock infiltration/polymerization before starting your freeze-substitution experiment. Specifically, set up the AFS, and at low temperatures, try to polymerize a mold filled with resin.

21. Glycine washes are typically used to block unreacted aldehyde groups in sections containing tissues fixed in aldehydes, therefore, this step may be treated as optional if the above fixation protocol (**Subheading 3.2.3.**) is used.

22. The specificity of staining can be judged based on the signal-to-noise ratio where noise is defined as the density of gold particles when no primary antibody is used, when a preimmune sera is used as the primary antibody, within an area of the section that contains no worm tissue, or within a tissue of worm in which it is known the antigen is not expressed. A local significant increase in the density of gold particles above background signifies the distribution of the labeled protein. If background staining is high, repeat the staining experiment but vary blocking conditions, the concentration of primary antibody, and/or the concentration of secondary antibody. If background staining persists, try other postembedding immunostaining protocols *(27 ,29,30)*, which may result in decreased background staining.

References

1. White, J. G., Southgate, E., Thomson, J. N., and Brenner, S. (1986) The structure of the nervous system of the nematode *Caenorhabditis elegans*. *Phil. Trans. R. Soc. Lond.* **B314,** 1–340.
2. Albertson, D. G. and Thomson, J. N. (1976) The pharynx of *Caenorhabditis elegans*. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **275,** 299–325.

3. Epstein, H. F., Waterston, R. H., and Brenner, S. (1974) A mutant affecting the heavy chain of myosin in *Caenorhabditis elegans*. *J. Mol. Biol.* **90,** 291–300.

4. Ward, S., Thomson, N., White, J. G., and Brenner, S. (1975) Electron microscopical reconstruction of the anterior sensory anatomy of the nematode *Caenorhabditis elegans*. *J. Comp. Neurol.* **160,** 313–337.

5. Waterston, R. H., Epstein, H. F., and Brenner, S. (1974) Paramyosin of *Caenorhabditis elegans*. *J. Mol. Biol.* **90,** 285–290.

6. Bosher, J. M., Hahn, B. S., Legouis, R., et al. (2003) The *Caenorhabditis elegans vab-10* spectraplakin isoforms protect the epidermis against internal and external forces. *J. Cell Biol.* **161,** 757–768.

7. Dernburg, A. F., McDonald, K., Moulder, G., Barstead, R., Dresser, M., and Villeneuve, A. M. (1998) Meiotic recombination in *C. elegans* initiates by a conserved mechanism and is dispensable for homologous chromosome synapsis. *Cell* **94,** 387–398.

8. Favre, R., Cermola, M., Paiva Nunes, C., Hermann, R., Muller, M., and Bazzicalupo, P. (1998) Immuno-cross-reactivity of CUT-1 and cuticlin epitopes between *Ascaris lumbricoides*, *Caenorhabditis elegans*, and *Heterorhabditis*. *J. Strut. Biol.* **123,** 1–7.

9. Favre, R., Hermann, R., Cermola, M., Hohenberg, H., Muller, M., and Bazzicalupo, P. (1995) Immuno-gold-labelling of CUT-1, CUT-2 and cuticlin epitopes in *Caenorhabditis elegans* and *Heterorhabditis sp.* processed by high pressure freezing and freeze-substitution. *J. Submicrosc. Cytol. Pathol.* **27,** 341–347.

10. Howe, M., McDonald, K. L., Albertson, D. G., and Meyer, B. J. (2001) HIM-10 Is required for kinetochore structure and function on *Caenorhabditis elegans* holocentric chromosomes. *J. Cell Biol.* **153,** 1227–1238.

11. Kirkham, M., Muller-Reichert, T., Oegema, K., Grill, S., and Hyman, A. A. (2003) SAS-4 is a *C. elegans* centriolar protein that controls centrosome size. *Cell.* **112,** 575–587.

12. Koppen, M., Simske, J. S., Sims, P. A., et al. (2001) Cooperative regulation of AJM-1 controls junctional integrity in *Caenorhabditis elegans* epithelia. *Nat. Cell Biol.* **3,** 983–991.

13. Leapman, R. D., Kocsis, E., Zhang, G., Talbot, T. L., and Laquerriere, P. (2004) Three-dimensional distributions of elements in biological samples by energy-filtered electron tomography. *Ultramicroscopy* **100,** 115–125.

14. MacQueen, A. J., Colaiacovo, M. P., McDonald, K., and Villeneuve, A. M. (2002) Synapsis-dependent and -independent mechanisms stabilize homolog pairing during meiotic prophase in *C. elegans*. *Genes Dev.* **16,** 2428–2442.

15. Rappleye, C. A., Paredez, A. R., Smith, C. W., McDonald, K. L., and Aroian, R. V. (1999) The coronin-like protein POD-1 is required for anterior-posterior axis formation and cellular architecture in the nematode *Caenorhabditis elegans*. *Genes Dev.* **13,** 2838–2851.

16. Rolls, M. M., Hall, D. H., Victor, M., Stelzer, E. H., and Rapoport, T. A. (2002) Targeting of rough endoplasmic reticulum membrane proteins and ribosomes in invertebrate neurons. *Mol. Biol. Cell.* **13,** 1778–1791.

17. Rostaing, P., Weimer, R. M., Jorgensen, E. M., Triller, A., and Bessereau, J. L. (2004) Preservation of immunoreactivity and fine structure of adult *C. elegans* tissues using high-pressure freezing. *J. Histochem. Cytochem.* **52,** 1–12.

18. Williams-Masson, E. M., Heid, P. J., Lavin, C. A., and Hardin, J. (1998) The cellular mechanism of epithelial rearrangement during morphogenesis of the *Caenorhabditis elegans* dorsal hypodermis. *Dev. Biol.* **204,** 263–276.

19. Yeh, E., Kawano, T., Weimer, R. M., Bessereau, J. L., and Zhen, M. (2005) Identification of genes involved in synaptogenesis using a fluorescent active zone marker in *Caenorhabditis elegans. J. Neurosci.* **25,** 3833–3841.

20. Gilkey, J. C. and Staehelin, L. A. (1986) Advances in ultrarapid freezing for the preservation of cellular ultrastructure. *J. Electron Micros. Tech.* **3,** 177–210.

21. Brüggeller, P. and Mayer, E. (1980) Complete vitrification in pure liquid water and dilute aqueous solutions. *Nature* **288,** 569–571.

22. Dubochet, J. and McDowall, A. W. (1981) Vitrification of pure water for electron microscopy. *J. Microsc.* **124,** RP3–RP4.

23. Al-Amoudi, A., Chang, J. J., Leforestier, A., et al (2004) Cryo-electron microscopy of vitreous sections. *Embo. J.* **23,** 3583–3588.

24. Walther, P. (2003) Recent progress in freeze-fracturing of high-pressure frozen samples. *J. Microsc.* **212,** 34–43.

25. McDonald, K. (1997) Freeze substitution. In: *Cells: A Laboratory Manual*, (Spector, D. L., Goldman, R. D., and Leinwand, L. A., eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 130.1–130.7.

26. Steinbrecht, R. A. and Muller, M. (1987) Freeze-substitution and freeze-drying. In: *Cryotechniques in Biological Electron Microscopy*, (Steinbrecht, S. R. and Zierold, K., eds.), Springer-Verlag, Berlin, 149–172.

27. Erickson, P. A., Geoffrey, P. L., and Fisher, S. K. (1993) Postembeding immuno-cytochemical techniques for light and electron microscopy. In: *Methods in Cell Biology: Antibodies in Cell Biology*, (Asai, D. J., ed.), Academic Press, Inc, San Diego, CA, pp. 283–310.

28. Brenner, S. (1974) The genetics of *Caenorhabditis elegans. Genetics* **77,** 71–94.

29. Hayat, M. A. (1989) *Colloidal Gold: Principles, Methods, and Applications,* Academic Press, San Diego, CA.

30. Verkleij, A. J. and Leunissen, J. L. M. (1989) *Immuno-Gold Labeling in Cell Biology,* CRC Press, Boca Raton, FL.

31. McDonald, K. (1999) High-pressure freezing for preservation of high resolution fine structure and antigenicity for immunolabeling. *Methods Mol. Biol.* **117,** 77–97.

32. Walther, P. and Ziegler, A. (2002) Freeze substitution of high-pressure frozen samples: the visibility of biological membranes is improved when the substitution medium contains water. *J. Microsc.* **208,** 3–10.

33. Giddings, T. H. (2003) Freeze-substitution protocols for improved visualization of membranes in high-pressure frozen samples. *J. Microsc.* **212,** 53–61.

34. Zhen, M. and Jin, Y. (1999) The liprin protein SYD-2 regulates the differentiation of presynaptic termini in *C. elegans. Nature* **401,** 371–375.

35. Li, X. and Sze, H. (1999) A 100 kDa polypeptide associates with the V0 membrane sector but not with the active oat vacuolar H(+)-ATPase, suggesting a role in assembly. *Plant J*. **17,** 19–30.

16

Intracellular pH Measurements In Vivo Using Green Fluorescent Protein Variants

Keith Nehrke

Summary

Whether by patch-clamp techniques or the use of fluorescent vital dyes, measurements of transepithelial ion flux in mammals are limited by cell accessibility. Furthermore, redundant functions and complex regulatory mechanisms can mask loss-of-function phenotypes through compensatory mechanisms. In this chapter, we present a technique whereby the optically transparent nematode *Caenorhabditis elegans*, engineered to express a fluorescent pH indicator protein, can be used to study how intracellular pH (pHi) fluctuates in response to environmental and/or experimental challenge. By using a live whole animal model, systemic, and even behavioral relationships to individual cellular pHi can be inferred. In combination with dye loading of excised or cultured cells, this technique also provides a powerful means of contrasting these relationships to biophysical measurements of ion flux.

Key Words: pH; pHluorin; ion transport; *C. elegans*; fluorescence.

1. Introduction

The movement of water, electrolytes, and nutrients through the cellular membrane is a dynamic process controlled by a variety of transport proteins. Our ability to measure the activity of these proteins relies on both knowledge of their substrates and an ability to isolate their function from other transport processes with similar substrate specificities. This has traditionally resulted in the study of membrane biophysics using isolated cells, where a reduced complexity and the ease of use of functional inhibitors are decided advantages over working with an intact organism. In general, progress in this field has been largely driven by technological innovation. Much of the original biophysical work utilized exogenous radiolabel that was taken up into the cell cytoplasm, into artificially prepared membrane vesicles, or selectively partitioned across

an intact epithelial sheet. With the advent of patch-clamp techniques, it became possible to measure electric currents resulting from the movement of charged molecules across a membrane, and even to observe the electric activity of ion transport at single molecule resolution. In the last 80 yr, however, fluorescent measurement techniques have expanded the scope of our ability to look into the cell; vital dyes such as MQAE, Fura-2, and BCECF have been developed to follow chloride *(1)*, intracellular calcium *(2)* and, in particular with regards to this report, intracellular pH (pHi) *(3)*. Correspondingly, increased temporal and spatial resolution of intracellular ion composition has followed, and changes in ion concentrations are routinely visualized in real time or in "microdomains" of the cell.

Many of the results obtained using these techniques form the basis of our understanding of membrane transport processes. Nonetheless, all have drawbacks, as well. For example, targeting and retention of small-molecule fluorescent indicators can prevent long-term studies in vivo, and they are difficult to load specifically into organelles to study subcellular ion transport mechanisms. To combat these problems, in recent years, second generation reporters have been designed that are both noninvasive and biocompatible. These reagents are largely based on fluorescent proteins isolated from *Aequorea Victoria*, and can be targeted to subcellular locations. The study of intact, living organisms expressing these proteins in specific cells (or cellular compartments) allows us a unique insight into how membrane transport processes are regulated in their native environment, where cell–cell interactions and systemic cell signaling occur normally. The inevitable drawback of this technology is that it is not readily adaptable to the biophysical study of membrane transport processes; the organism itself provides the physiological environment and we, as researchers, are limited to observation. This drawback not withstanding, these novel reagents have advantages, as well. Experiments that were not feasible when studying isolated cells become possible, such as identifying how changes in cellular electrolyte content influence behavior. In particular, these types of technologies are especially adaptable to a small, transparent organism with a limited number of cells and a defined repertoire of behaviors such as *Caenorhabditis elegans*. Our focus here will be on the use of fluorescent pH-indicator proteins in nematodes.

The control of pHi is an important facet of cellular homeostasis. Changes in the resting pHi of a cell such as occur in response to hormonal stimulation or during metabolic acidosis can cause profound alterations in cellular architecture and enzyme activity, which may in turn lead to functional consequences for the organism. In addition, pHi regulation may be important for cell volume control, sustained secretion, nutrient uptake, and cell migration and signaling (for review, *see* **ref. *4***). Our ability to measure changes in pHi under physi-

ological conditions in worms is, like many of the reporter systems available, based upon the fluorescent properties of green fluorescent protein (GFP). Both the absorbance and emissions of GFP are pH-sensitive *(5,6)*, and variants of GFP that have increased or reduced pKas relative to the original wild-type protein *(7–9)* have been used to follow intracellular or organelle pH, mainly in cell culture applications *(10,11)*. These techniques as a rule measure changes in absolute fluorescent intensity of the biosensor, and are applicable for samples where movement is minimal or where quantum changes in fluorescent intensity can be used to measure isolated events. However, several GFP variants have been described that are suitable for ratiometric imaging techniques *(12,13)*. Ratiometric probes have multiple excitation or emission maxima that show opposing changes in fluorescence with pH, and provide an advantage over conventional GFP owing to a decreased sensitivity to the indicator concentration, photobleaching, and changes in the pathlength. One such biosensor is the pH-sensitive fluorophore "pHluorin" *(12)*. pHluorin was originally targeted to neuronal vesicles to follow pH changes that accompany synaptic fusion events *(12)*, and has since been used to study endo- and exocytosis *(14–18)*. pHluorin has also been expressed in the nematode intestine to examine how nutrient uptake modulates cytoplasmic pH *(19)*, to oocytes to highlight pH changes that occur during fertilization (K. Nehrke, unpublished data), and to mitochondria to examine organelle breakdown during erythrocyte maturation *(20)*. The powerful genetic and reverse genetic techniques available in *C. elegans* make it an ideal model system for using pHluorin to study the contribution of individual ion transport proteins to pH homeostasis and to correlate deficits in pH homeostasis with loss-of-function phenotypes.

Alternate, parallel approaches also have become recently available for excising cells or tissues from the worm to work with in isolation. These types of approaches include techniques such as embryonic cell culture *(21,22)* and ex vivo patch clamp *(23–29)*, which allow direct access to the cells being studied and permit the use of conventional biophysical methodologies. In combination with in vivo imaging, these techniques facilitate obtaining a truly "integrated" picture of membrane transport processes and their underlying molecular components in a physiological setting.

2. Materials

1. pH-sensitive GFP expression vector.
2. 2% Agarose pads.
3. Nexaband S/C cyanoacrylate glue (GluStitch Inc., Point Roberts, WA).
4. Superfusate: 135 mM NaCl, 5.4 mM KCl, 0.4 mM KH$_2$PO$_4$, 0.33 mM NaH$_2$PO$_4$, 20 mM HEPES, 1.2 mM CaCl$_2$, 0.8 MgSO$_4$, pH adjusted to 7.4 with Tris-base immediately prior to use.

5. Calibration solution: 135 mM KCl, 2 mM K$_2$HPO$_4$, 20 mM HEPES, 1.2 mM CaCl$_2$, 0.8 mM MgSO$_4$, pH adjusted to between 5.4 and 8.4 using HCl or KOH. Add nigericin to 20 µm prior to use.
6. Perfusion chamber (Warner Instruments, Hamden, CT).
7. Fluorescent microscope, monochromator or light source/filter wheel, and charge-coupled device (CCD) camera.
8. Computer, framegrabber, and acquisition/analysis software.
9. Fluorescent pH-sensitive vital dye BCECF-AM (Molecular Probes, Eugene, OR).

3. Methods

The methods described next outline (1) the construction of pH-sensitive GFP (pHluorin) expression vectors for use in worms, (2) the preparation of agarose pads and glue-down techniques, (3) dual-excitation ratio imaging, and (4) *in situ* pH calibration.

3.1. pH-Sensitive GFP Expression Plasmids

Most GFP variants are sensitive to pH, although with different spectral characteristics and pKas. This sensitivity results from a conformational change following protonation of the chromophore and can be influenced by amino acid substitutions that modulate the conformational switching or that influence protonation through electrostatic interactions. In our laboratory, we have mainly employed pHluorin *(12)* as a pH sensor, owing to the fact that movement of the worm can be a confounding factor when using glue-down techniques, making ratiometric imaging a necessity. Two pHluorin variants were originally derived in Dr. James Rothman's laboratory via combinatorial mutagenesis of the wild-type GFP. The "ratiometric" variant exhibits a bimodal excitation spectrum with a reversible excitation ratio change between pH 5.5 and 7.5, whereas the "ecliptic" variant looses fluorescence as the pH diminishes and is, thus, nearly invisible below pH 6.0. The ecliptic variant is also available with a characteristic S65T mutation found in enhanced GFP, and this "supercliptic" protein is much brighter than either of the originals. Although at least six mutations are known to exist in each variant, the minimal sets of mutations that confer particular attributes and pH sensitivity have not been delineated (**Table 1**). Our laboratory has generated a wide range of vectors for use in *C. elegans* that express both the ecliptic and ratiometric pHluorin variants (courtesy of Drs. James Rothman and Gero Missenboeck, Memorial Sloan-Kettering Cancer Center [MSKCC], New York, NY) driven by a variety of cell specific promoters, to which protein targeting signals can be added (*see* **Note 1**). The vector backbones are based upon pFH6.II (courtesy of F. Hagen, University of Rochester, Rochester, NY), which is a derivative of the Fire lab vector pPD95.81 (courtesy of A. Fire, Carnegie Institute of Washington, Baltimore, MD). The pHluorin expression vectors contain four synthetic introns and either the *unc-54* or *clh-3b* 3'-UTR (the latter for expression in germ cells), as shown in **Fig. 1**.

Table 1
Examples of Green Fluorescent Protein Variants Used as Biosensors for Measuring pH

Common name	Mutations	Reference	Spectral properties Excitation | emission (nm | nm)	pKa	Notes
• Ecliptic (or Synapto) pHluorin	S147D, N149Q, T161I, S202F, Q204T, A206T	*12*	475 | 535	7.11	Eclipsed as pH decreases
• Ratiometric pHluorin	E132D, N147E, N149L, N164I, K166Q, I167V, R168H, S202H	*12*	410/470 | 535	7.23	Dual-excitation ratio-metric indicator
• EGFP	F64L, S65T	*7*	488 | 510	5.98	Common variant
• phGFP	S65T, H148D	*9*	488 | 510	7.95	Clean isosbestic point at 435 nm may allow dual excitation measurements
• EYFP	S65G, V68L, S72A, T203Y	*9*	510 | 535	7.00	Common variant
• deGFP4	C48S, S65T, H148C, T203C	*13*	400 | 460/515	7.37	Dual-emission sensor

EGFP, enhanced green fluorescent protein; phGFP, pH-sensitive green fluorescent protein; EYFP, enhanced yellow fluorescent protein; deGFP, dual-emission green fluorescent protein.
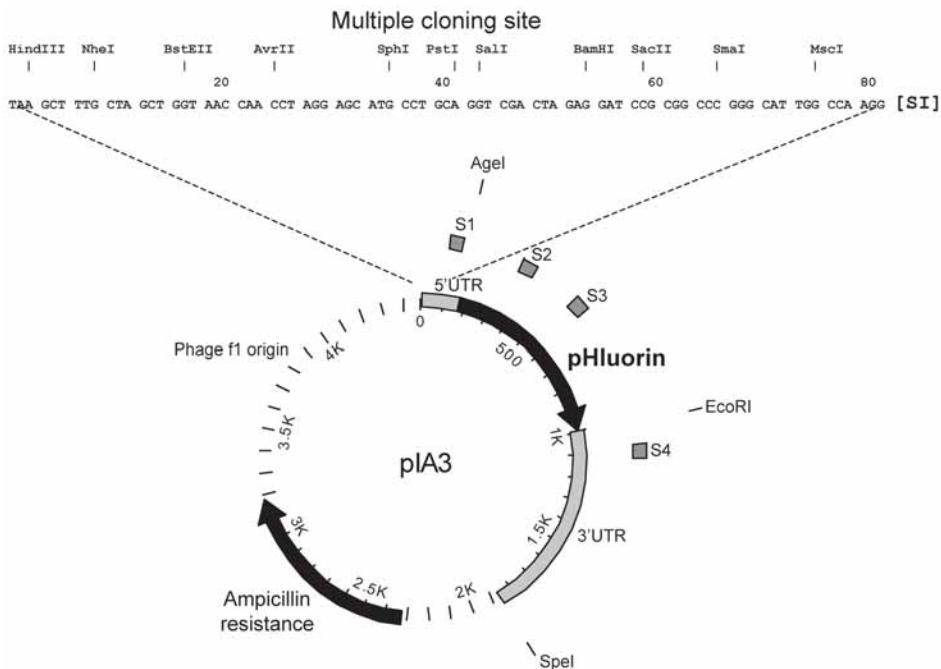
Fig. 1. Schematic drawing of pIA3, a promoterless construct for expressing ratiometric pHluorin in worms. SI, synthetic intron.

To obtain these vectors, requests should be sent first to Dr. Gero Miesenbock at MSKCC. Once a material transfer agreement is in place, MSKCC will forward the information to our laboratory and we will make any of our reagents available. These vectors can be injected and integrated using conventional techniques; we have not noted any phenotypic or behavioral changes resulting from the expression of pHluorin in any of the transgenic strains that we have examined.

3.2. Preparation of Agarose Pads and Glue-Down Techniques

Transgenic worms with cell specific pHluorin expression are immobilizing for viewing under perfusion by gluing them onto an agarose pad, as described for Ca^{2+} imaging using the cameleon biosensor *(30)*. The pads are generally formed from 2% agarose in M9 media; it is essential that water alone not be used, as the divalent cations in the pad accelerate polymerization of the cyanoacrylate adhesive. In addition, the tonicity of the final agarose preparation is important, and should mimic the tonicity of the superfusate to avoid osmotic imbalance and salt-related effects such as swelling or shrinkage of the worm. Depending on the physiological function to be assayed, we will place well-fed worms on an

unseeded plate for 1 h prior to mounting them to allow expulsion of the immediate contents of the intestinal lumen.

3.2.1. Pouring the Agarose Pad

1. Melt 1 g of agarose in 50 mL of M9 solution. Allow the agarose to cool slightly. It may be convenient to maintain the melted agarose in a heating block at 65°C for later use.
2. Place approx 8 μL of the melted agarose onto clean 50-mm cover glass (0.17 mm optical quality). Working quickly, before the agarose hardens, place a second 40-mm cover glass on top of the first to form a small, thin patch. Avoid bubbles. Slide this patch gently using the top piece of cover glass so that it will be positioned in the center of the viewing area when the 50-mm cover glass is mounted to form the bottom of the perfusion chamber.
3. Pry the top cover glass off the pad, taking care that the pad remains firmly attached to the bottom cover glass. Then, use a small, flame-drawn capillary to quickly apply a thin coating of Nexaband S/C cyanoacrylate glue to the edges of the pad. This will prevent the pad from floating upward during perfusion.

3.2.2. Gluing the Worm to the Pad

1. Working quickly, use a worm pick to move a small amount of bacterial lawn to the agarose (to encourage the worm to crawl off the pick), then move the worm itself onto the pad. Take special care not to break through the pad with the pick during the transfer, as this will result in the pad lifting during imaging. A longer, slightly rounded pick will work better than a wide, flat pick.
2. Apply a small amount of Nexaband S/C veterinary adhesive to the worm using a patch pipet or flame drawn capillary with the tip broken off. The adhesive polymerizes upon contact with divalent cations in the pad, and is ideal for immobilizing worms for viewing under superfusate over a prolonged time period. Use the smallest amount of adhesive that results in immobilization, as excess adhesive will dehydrate the worm. The butyl derivative appears to be more biocompatible and, to avoid autofluorescence, the clear adhesive should be used. The slightest touch of the capillary to the worm should result in enough adhesive to prevent motion. More extensive contact may allow polymerization of the adhesive within the capillary itself.
3. Immediately place a small droplet of superfusate on the worm to prevent dehydration. Serotonin can be included at 2 m*M* to stimulate pharyngeal pumping, as necessary. Use the cover glass to form the bottom of a perfusion chamber, with the worm centered and facing upward. A small amount of silicone grease applied to the bottom of the chamber will help form a seal. The chamber is then moved to a mounting platform, where it is secured and placed onto a microscope.

3.3. Dual-Excitation Ratio Imaging

Although many types of microscopes will work, our preference is an inverted differential interference contrast microscope with dual camera ports and a long

working distance condenser. The objectives should be of the best fluorescence quality affordable and capable of imaging through both a cover slip and agarose pad (0.17 mm each). Although we routinely use a ×20 dual-emmersion objective (NA = 1.3) for obtaining images of the intestine, we also use a ×60 long working distance dry objective with a lower numerical aperture for examining single cells. Dichroic and emission filters are generally available from the microscope manufacturer as application-specific sets, and the most commonly used glass inserts are probably made by Chroma Technology Corp. (Rockingham, VT), with filter sets specifically designed for pHluorin imaging. As a light source, we have several PolyChromeIV monochromators from TILL Photonics (Germany) that provide high intensity illumination from 320 to 680 nm and can change wavelengths at greater than 400 nm/ms. However, there are a variety of other systems available, including the Lambda DG-4 (Sutter Instruments, Novato, CA). **Table 2** is a list of related reagents, equipment, and manufacturers websites; although this list is not comprehensive, it should provide a starting point for setting up a quality-imaging rig.

One alternative to a monochromator is a high-speed filter wheel, and there are a number of filter wheel manufacturers including Sutter (Novato, CA) and Ludl (Hawthorne, NY). In either case, an invaluable option is an electronic shutter, which can be used to reduce photobleaching between frame acquisitions. However, the changes in wavelength are not nearly as fast using a filter wheel as a monochromator. This can become a problem with dual-excitation ratiometric techniques, as the sample may be moving, and the images acquired at each wavelength may not align. However, if speed is not an issue (for example, if the nematode has been anesthetized), then this type of system can be much more cost effective than a monochromator. The X-Cite 120 illuminator (EXFO, Canada) is ideal for generating GFP excitation wavelengths with this type of system.

In terms of a camera, speed is more important than resolution and color is not strictly necessary. We use a PCO Sensicam VGA cooled digital camera, but there are a wide variety of options available. We recommend a slow scan interline transfer monochromatic camera with 640×480 pixels (10 μM each) of resolution, high quantum efficiency in the GFP spectra, and electronic cooling to ensure an even signal distribution over the entire CCD. To link all of these tools together, we employ the latest in PC technology, a PCI frame grabber from TILL Photonics, and either TILLvisION or AXON Imaging Workbench software displayed on a large flat screen LCD monitor. One advantage to the AXON software is that it allows you to "shift on the fly;" that is, you can change the acquisition rate or length of an experiment while that experiment is still running. In contrast, with the TILLvisION software, a protocol is compiled in an external processor to enhance temporal resolution, and once the protocol is running, you either let it play out or break it, with no in between.

Table 2
Manufacturers' and Suppliers' Websites [a]

Company name	Website	Product
Bioscience tools	www.biosciencetools.com	Instrumentation and supplies
Warner Instruments	www.warneronline.com	Instrumentation and supplies
World Precision Instruments	www.wpiinc.com	Instrumentation and supplies
Ludl Electronic Products	www.ludl.com	Instrumentation
Sutter Instrument Company	www.sutter.com	Instrumentation
Hamamatsu Photonics	www.usa.hamamatsu.com	Cameras
PCO	www.pco.de	Cameras
Roper Princeton Scientific	www.roperscientific.com	Cameras
Chroma Technology Corp.	www.chroma.com	Optical filters
EXFO	www.exfo-lifesciences.com	Instrumentation and illumination (X-Cite 120)
Applied Scientific Instrumentation	www.asimaging.com	Imaging solutions
AXON Instruments	www.imagingworkbench.com	Instrumentation and software (Imaging Workbench)
TILL Photonics	www.till-photonics.com	Instrumentation and software (TILLvisION)
Scanalytics	www.scanalytics.com	Software (IP Lab)
Universal Imaging	www.universal-imaging.com	Software (Metafluor)
Molecular Probes	www.probes.invitrogen.com	Fluorophores
TefLabs	www.teflabs.com	Fluorophores
Microscopy Online	www.microscopy-online.com	General information
The Photonics Directory	www.photonics.com	General Information
GFP applications page	www.yale.edu/rosenbaum/gfp_gateway.html	Information on GFP applications

[a] The nature of imaging protocols often requires tailoring equipment to a specific need, but there may be numerous means to achieve the same end. Therefore, while this is not meant to be a comprehensive list, it should serve as a starting point for researching a cost-effective imaging solution.

However, there are a large number of software tools that can run fluorescent image acquisition protocols, so the most important considerations are first, whether a program supports your hardware choices; and second, how sophisticated a protocol and/or analysis will be necessary for your application. Other considerations include the amount of space that experimental files occupy on the hard drive and the ease of manipulating or exporting data files in cross-platform compatible formats. Finally, existing systems that are capable of static fluorescent imaging can often be easily adapted to ratiometric imaging of moving samples for a fraction of the cost of a new system.

3.3.1. Setting the Acquisition Parameters

1. Determine an exposure time. All of the protocols in this section need to be performed in a closed-off area where ambient light is kept to a minimum. In order to keep from bleaching both the CCD and/or the sample, an appropriate exposure time needs to be established. *It is also extremely important not to view the sample under fluorescent light.* This will lead to rapid photobleaching, and subsequently fluorescent emission ratios will not accurately reflect the pH. Positioning the sample can be done using transmitted light, and a single-frame fluorescent image can then be acquired at each of the relevant wavelengths. For ratiometric pHluorin, these exposures will occur at excitation wavelengths of 410 and 470 nm (generally using a 15-nm window centered around these wavelengths), and the emissions will be measured in a region centered around 535 nm (with a 50-nm window). Each exposure should be long enough to provide sufficient linear resolution that a ratio of the emissions at each wavelength is capable of reflecting small changes in pH. For example, the dynamic range of 12-bit data is 4096 grayscale levels. The more of this scale we use (until we saturate the CCD), the greater our ability to measure precise emission ratio changes, and the "smoother" the data will appear. In practice, however, the advantage of increased precision with longer exposure times is more than offset by increased photobleaching. Therefore, our aim is generally to achieve several hundred grayscale levels of signal over background on each channel. Obviously, data that is acquired under exposure conditions where the CCD is saturated on either channel cannot be used, and therefore, it's important to remember that the emissions may change as a result of changes in pHi. Do not set the initial exposure time so high that the signal is at the upper end of the dynamic range of the CCD, or small pH-dependent increases in the emission intensity may result in saturation. With our system, generally we find that between 10- and 50-ms exposures at each wavelength are appropriate for a variety of transgenic strains.

2. Determine binning size. There are two types of background; the first of these is "electronic noise," which is contributed by the camera, whereas the second is autofluorescence that results from the pad or the worm itself. Binning is a technique whereby the signal from adjacent pixels can be combined into a single value. The advantages of binning are that it increases the signal per unit area and generally allows for an increased signal-to-noise ratio. This can be partially attributed

to the fact that only the "real" signal is amplified, including the "real" background, but the "electronic noise" component of the signal is not amplified. Binning can greatly improve the quality of a low-signal intensity sample when there is very little background signal, and can result in decreased exposure times and photobleaching. The disadvantages are that it causes a decreased resolution and often effectively reduces the dynamic range. You might consider binning if photobleaching or signal-to-noise ratios are issues during acquisition, but this is generally an empirical determination. Whatever the choice, it is important to maintain identical acquisition parameters throughout the experiment and to use those parameters when performing an *in situ* calibration (*see* **Note 1**).

3.3.2. Acquiring and Analyzing Data

1. For real-time observation of physiological changes in pH, begin by ensuring that the sample is sufficiently immobilized that it remains in the field of view and in focus during constant perfusion. Two common problems at this point are first, that the worm becomes unglued and second, that the pad floats. These problems can usually be avoided by preceding the actual experiment with a trial run using the established acquisition parameters. We allow at least 10 min on the pad prior to initiating our protocol, and we will acquire ratio images at 2 Hz during that time. This time period is also useful to determine a baseline resting pH and to allow drugs time to act. The glue-down prep appears to be viable for at least 1 h in our hands.

2. When initiating the protocol, timing is everything. Changes in pHi can occur slowly or quickly, depending on the process being studied. In order to couple solution changes with acquisition frequency, we use electronic pinch valves that are controlled by TTL pulses, and we set up protocols that generate pulses designed to switch superfusate solutions in parallel with increases or decreases in the rate of image acquisition. This allows us to further reduce photobleaching during periods of relative inactivity, but provides increased temporal resolution for following rapid changes in pHi. It also allows us to automate the acquisition process.

3. After having immobilized the worm under superfusate, determining the exposure time, binning, and how best to subtract the background fluorescence, as well as optimizing/synchronizing the frame acquisition rate with changes in superfusate, the actual experiment is trivial; the software runs your protocol, while you ensure that the worm does not move out of focus (generally by accompanying the protocol with a live viewing window). Out-of-focus images will generally have a low apparent pHi.

4. Generate 410/470 ratio images. Our software determines these ratios on a pixel-by-pixel basis, using background-subtracted values, then displays the results as a pseudo-color heat map. In order to reduce the contribution of out-of-the-focal-plane fluorescence, thresholding can be used so that weak signals (at either 410 or 470 nm) are set to a zero value on the ratio map. The ratio map should resemble the fluorescent expression pattern, except that that the ratio will be nearly equivalent within each cell, independent of fluorescent expression intensity (**Fig. 2**).
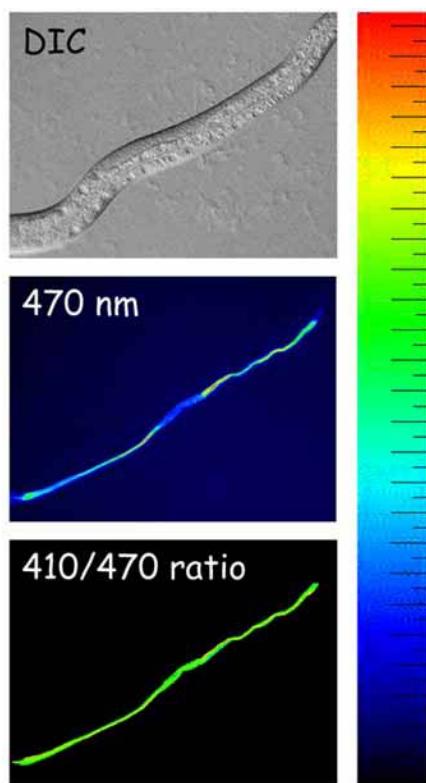
Fig. 2. The apparent pH, as reflected by the 410/470 nm excitation ratio (×1000) of transgenic pHluorin targeted to the apical membrane of the intestine, is uniform despite differences in transgene expression levels and out-of-focus fluorescence observed at 470 nm.

If this is not the case, then the background selection may be inappropriate. Generally, when analyzing a time-delimited image stack such as you would acquire from a real-time experiment, we link the background to each individual image in the stack to control for fluctuations in the excitation intensity or camera background. In this case, it is important to make sure that the worm has not moved into the area that you have defined as your background at any point during the experiment.

5. Generate a kinetic for each region of interest in the ratio map. Select regions of the worm to follow pHi using drawing tools to highlight individual cells or organs (*see* **Note 2**). Ratios obtained from these areas can be converted to pHi using the Boltzmann equation following an *in situ* calibration as described in **Subheading 3.4.** The technical graphing software Origin 7.5 (Originlab) is useful for transforming ratio data to apparent pHi.

3.4. **In Situ** *Calibration*

A final control at this point is to ensure that the reporter accurately reflects pHi. In the case of cytoplasmic pHluorin, this is done using an *in situ* calibration via exposure to superfusate containing high K+/nigericin where pHo = pHi *(31)*. As might be expected, in order to calibrate pH-to-fluorescent ratio, the cells must first be excised from the worm. Generally, exposing the cells is as simple as cutting a slit in the cuticle as close to the cell of interest as possible using a 30-gage needle. Often, following data acquisition, we will gently slice the sample open under a low-power objective, then proceed with the calibration. It may help to do this under slightly hypertonic conditions (using sucrose to adjust the osmolarity of the calibration solution) so that the cells will not burst. Once the cells are exposed, the pH is raised from 5.8 to 8.4 in 0.2 U steps using preadjusted high K+/nigericin calibration solution, and at each step the 410/470 nm fluorescent ratio is acquired. Finally, pHo (*x*) is plotted vs the ratio (*y*) and the resulting sigmoidal curve is fitted to the following:

$$y = \frac{A1 - A2}{1 + e^{(x-x_0)/dx}} \qquad \text{Boltzmann equation,}$$

where *A1* and *A2* represent the limits of the 410/470 nm fluorescent ratio at infinitely low or high pHs, x_o is the pH midpoint at (A1+A2)/2, and *x* is the observed pH (*see* **Fig. 3** for a sample curve). The constants derived from this fitting are then used to transform the emission ratios obtained experimentally to pH values. It is important to remember that the acquisition parameters used to obtain data for fitting to the Boltzmann equation should be the same as the parameters used to acquire experimental data, and sample excitation should be minimized to avoid photobleaching (we allow 3 min at each pHo for the cells to equilibrate, then obtain three successive ratio images). Deviations from this regiment can greatly decrease the reliability of the data, especially at emission ratios near A1 and A2 (corresponding to pH values at the extreme ends of the physiological scale), and may preclude comparisons of data obtained using different settings.

4. Notes

1. A wide range of protein targeting signals can be added to pH indicator proteins; for example, the superecliptic pHluorin has been used for measuring synaptic events where a quantum change in pH occurs during vesicle fusion *(12)*. However, motion artifacts may pose a problem with using superecliptic pHluorin in a live animal, though this variant has recently been used to image fusion events during odorant stimulation of individual glomeruli in mice *(32)*. On the other hand, the pKa of ratiometric pHluorin (around 7.2) makes it difficult but not impossible to accurately measure pH at either low or high physiological values,
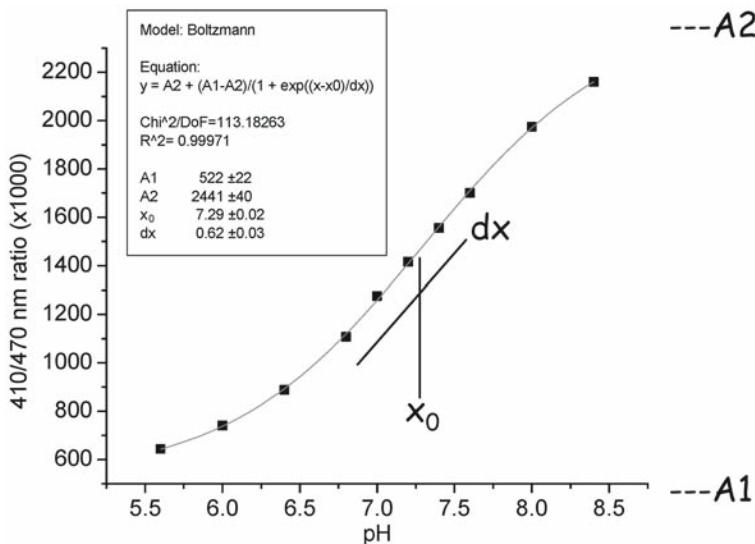
Fig. 3. Sample *in situ* calibration curve for ratiometric pHluorin. The ratio of the 535 nm emissions following 410/470 nm dual-excitation was plotted against the external pH under conditions where pHi = pHo, then fitted to a sigmoidal curve using the Boltzmann equation and Origin 7.5 software.

such as occurs in synaptic vesicles, endosomes, or mitochondria. In these instances, other nonratiometric variants (*see* **Table 1**) with pKa values closer to the organelle pH values may be more applicable.

2. Ratiometric dual-excitation imaging reduces errors compared with single wavelength measurements, but samples of different intensities can still have a different apparent pHi, especially at low signal-to-noise ratios. For this reason, the absolute fluorescent intensity should be kept as constant as possible when making comparisons among different samples.

3. Because of our inability to control the buffer composition inside of a worm, this technology is most useful in comparing the resting pHi of a cell with a measurable phenotype and assessing pHi fluctuations that accompany a physiological phenomenon. In addition, in vivo pH indicators are well suited for use with reverse genetic screening techniques to select for proteins that maintain pH homeostasis in various cells. Purely biophysical analyses of membrane transport characteristics and kinetics requires the ability to manipulate buffer compositions and conditions. In this regard, excised or embryonic cells can be studied using vital dyes in place of genetic indicators. BCECF is commonly used for measuring pHi and comes as a cell-permeant acetylmethylester that is loaded into the cells by addition to the superfusate. Upon cell entry, endogenous esterases cleave off the methylester substituent, effectively trapping the dye in the cell.

Two common sources for BCECF are Molecular Probes (Eugene, OR) and Tef-Labs (Austin, TX). Although the excitation wavelengths are different for BCECF and pHluorin, an identical dichroic/emission set can be used for both applications with the use of a monochromator as an excitation light source, thus saving the cost of a complete filter set.

Acknowledgments

References

1. Verkman, A. S., Sellers, M. C., Chao, A. C., Leung, T., and Ketcham, R. (1989) Synthesis and characterization of improved chloride-sensitive fluorescent indicators for biological applications. *Anal. Biochem.* **178,** 355–361.
2. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260,** 3440–3450.
3. Rink, T. J., Tsien, R. Y., and Pozzan, T. (1982) Cytoplasmic pH and free Mg2+ in lymphocytes. *J. Cell Biol.* **95,** 189–196.
4. Orlowski, J. and Grinstein, S. (2004) Diversity of the mammalian sodium/proton exchanger *SLC9* gene family. *Pflugers Arch.* **447,** 549–565.
5. Bokman, S. H. and Ward, W. W. (1981) Renaturation of Aequorea green-fluorescent protein. *Biochem. Biophys. Res. Commun.* **101,** 1372–1380.
6. Ward, W. W. and Bokman, S. H. (1982) Reversible denaturation of Aequorea green-fluorescent protein: physical separation and characterization of the renatured protein. *Biochemistry* **21,** 4535–4540.
7. Kneen, M., Farinas, J., Li, Y., and Verkman, A. S. (1998) Green fluorescent protein as a noninvasive intracellular pH indicator. *Biophys. J.* **74,** 1591–1599.
8. Llopis, J., McCaffery, J. M., Miyawaki, A., Farquhar, M. G., and Tsien, R. Y. (1998) Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins. *Proc. Natl. Acad. Sci. USA* **95,** 6803–6808.
9. Elsliger, M. A., Wachter, R. M., Hanson, G. T., Kallio, K., and Remington, S. J. (1999) Structural and spectral response of green fluorescent protein variants to changes in pH. *Biochemistry* **38,** 5296–5301.
10. Takahashi, A., Zhang, Y., Centonze, E., and Herman, B. (2001) Measurement of mitochondrial pH in situ. *BioTechniques* **30,** 804–808.
11. Matsuyama, S., Llopis, J., Deveraux, Q. L., Tsien, R. Y., and Reed, J. C. (2000) Changes in intramitochondrial and cytosolic pH: early events that modulate caspase activation during apoptosis. *Nat. Cell. Biol.* **2,** 318–325.

12. Miesenbock, G., De Angelis, D. A., and Rothman, J. E. (1998) Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature* **394,** 192–195.

13. Hanson, G. T., McAnaney, T. B., Park, E. S., et al. (2002) Green fluorescent protein variants as ratiometric dual emission pH sensors. 1. Structural characterization and preliminary application. *Biochemistry* **41,** 15,477–15,488.

14. Sankaranarayanan, S. and Ryan, T. A. (2001) Calcium accelerates endocytosis of vSNAREs at hippocampal synapses. *Nat. Neuroscience.* **4,** 129–136.

15. Ohara-Imaizumi, M., Nakamichi, Y., Tanaka, T., Katsuta, H., Ishida, H., and Nagamatsu, S. (2002) Monitoring of exocytosis and endocytosis of insulin secretory granules in the pancreatic beta-cell line MIN6 using pH-sensitive green fluorescent protein (pHluorin) and confocal laser microscopy. *Biochem. J.* **363,** 73–80.

16. Poskanzer, K. E., Marek, K. W., Sweeney, S. T., and Davis, G. W. (2003) Synaptotagmin I is necessary for compensatory synaptic vesicle endocytosis in vivo. *Nature* **426,** 559–563.

17. Mitchell, S. J. and Ryan, T. A. (2004) Syntaxin-1A is excluded from recycling synaptic vesicles at nerve terminals. *J. Neurosci.* **24,** 4884–4888.

18. Ashby, M. C., De La Rue, S. A., Ralph, G. S., Uney, J., Collingridge, G. L., and Henley, J. M. (2004) Removal of AMPA receptors (AMPARs) from synapses is preceded by transient endocytosis of extrasynaptic AMPARs. *J. Neurosci.* **24,** 5172–5176.

19. Nehrke, K. (2003) A reduction in intestinal cell pHi due to loss of the *Caenorhabditis elegans* Na+/H+ exchanger NHX-2 increases life span. *J. Biol. Chem.* **278,** 44,657–44,666.

20. Vijayvergiya, C., De Angelis, D., Walther, M., et al. (2004) High-level expression of rabbit 15-lipoxygenase induces collapse of the mitochondrial pH gradient in cell culture. *Biochemistry* **43,** 15,296–15,302.

21. Estevez, A. Y., Roberts, R. K., and Strange, K. (2003) Identification of store-independent and store-operated Ca^{2+} conductances in *Caenorhabditis elegans* intestinal epithelial cells. *J. Gen. Physiol.* **122,** 207–223.

22. Christensen, M., Estevez, A., Yin, X., et al. (2002) A primary culture system for functional analysis of *C. elegans* neurons and muscle cells. *Neuron* **33,** 503–514.

23. Bamber, B. A., Richmond, J. E., Otto, J. F., and Jorgensen, E. M. (2005) The composition of the GABA receptor at the *Caenorhabditis elegans* neuromuscular junction. *Br. J. Pharmacol.* **144,** 502–509.

24. Machaca, K., DeFelice, L. J., and L'Hernault, S. W. (1996) A novel chloride channel localizes to *Caenorhabditis elegans* spermatids and chloride channel blockers induce spermatid differentiation. *Dev. Biol.* **176,** 1–16.

25. Goodman, M. B., Hall, D. H., Avery, L., and Lockery, S. R. (1998) Active currents regulate sensitivity and dynamic range in *C. elegans* neurons. *Neuron* **20,** 763–772.

26. Richmond, J. E. and Jorgensen, E. M. (1999) One GABA and two acetylcholine receptors function at the *C. elegans* neuromuscular junction. *Nat. Neurosci.* **2,** 791–797.

27. Rutledge, E., Bianchi, L., Christensen, M., et al. (2001) CLH-3, a ClC-2 anion channel ortholog activated during meiotic maturation in *C. elegans* oocytes. *Curr. Biol.* **11,** 161–170.

28. Jospin, M., Mariol, M. C., Sâegalat, L., and Allard, B. (2002) Characterization of K(+) currents using an in situ patch clamp technique in body wall muscle cells from *Caenorhabditis elegans*. *J. Physiol.* **544,** 373–384.

29. Jospin, M. and Allard, B. (2004) An amiloride-sensitive H^+-gated Na^+ channel in *Caenorhabditis elegans* body wall muscle cells. *J. Physiol.* **559,** 715–720.

30. Kerr, R., Lev-Ram, V., Baird, G., Vincent, P., Tsien, R. Y., and Schafer, W. R. (2000) Optical imaging of calcium transients in neurons and pharyngeal muscle of *C. elegans*. *Neuron* **26,** 583–594.

31. Thomas, J. A., Buchsbaum, R. N., Zimniak, A., and Racker, E. (1979) Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. *Biochemistry* **18,** 2210–2218.

32. Bozza, T., McGann, J. P., Mombaerts, P., and Wachowiak, M. (2004) In vivo imaging of neuronal activity by targeted expression of a genetically encoded probe in the mouse. *Neuron* **42,** 9–21.

17

Automated Imaging of *C. elegans* Behavior

Christopher J. Cronin, Zhaoyang Feng, and William R. Schafer

Summary

Automated systems for recording and analyzing behavior have many applications for the study of neurobiology in *Caenorhabditis elegans*. In particular, machine-based approaches allow for precise quantitative definitions of behavioral phenotypes that have traditionally been subjectively described by individual observers. Automated systems also facilitate the analysis of behaviors that occur over long time scales or are difficult to detect by eye. Here we describe the detailed methodology for the use of one recently described automated tracking system for *C. elegans*. These protocols make it possible to measure a wide range of parameters related to the morphology, body posture, and locomotion patterns of individual wild-type and mutant nematodes.

Key Words: Behavior; machine vision; locomotion; automated tracking system.

1. Introduction

Caenorhabditis elegans is an attractive experimental organism for the analysis of behavior and development at the molecular and cellular levels. In particular, its simple and well-described nervous system makes it possible to use genetic analysis to functionally dissect the molecular mechanisms underlying poorly understood aspects of neuronal function and relate them to specific patterns of behavior. However, a long-standing limitation in the analysis of behavior in *C. elegans* has been the subjectivity and imprecision of behavioral assays. For example, mutations in more than 100 genes have been described that cause abnormal or uncoordinated movement. Standard descriptions of uncoordinated phenotypes such as "kinker" or "coiler" are inadequate to convey with precision the nature of a given locomotor defect. Even though mutants affecting a common molecular target generally have behavioral phenotypes that look similar to a trained observer viewing them side by side, it is difficult if not impossible to assess which mutants have genuinely similar phenotypes based on published

descriptions alone. In addition, some behavioral mutants, including many affecting important and conserved aspects of neurotransmission, have phenotypes that are subtle to the eye or are manifested over long time scales. Such phenotypes are very difficult for a human observer to score based on real-time observation.

One way these problems have been surmounted is through use of video capture and analysis systems. By recording and analyzing the behavior of individual animals, often for long time intervals, it is possible to rigorously identify and quantitate deviations from wild-type behavior that are difficult to discern by eye. A number of such systems have been described in the recent literature. Some of these systems record the behavior of multiple animals at lower magnification; these tend to measure a relatively small number of parameters related to gross locomotor behavior *(1,2)*. Others record single animals at higher magnification; these can quantify more detailed aspects of body movement and posture, but obviously are more limited in the ability to rapidly collect data on statistically significant numbers of animals *(3–7)*.

Here, we present a detailed description of the methodology for the use of one recently described machine vision system for analyzing *C. elegans* behavior *(6)*. It should be noted that this system is under continued development, and that some of the specific details of the described protocols are likely to change soon after the time of writing. For the most up-to-date protocols, the reader is directed to the relevant chapter in the frequently revised Internet resource, Wormbook. However, it is hoped that this chapter will provide the reader with a sense of the detailed methodology underlying the use of an automated tracking system to study worm behavior. It is hoped that these insights will be valuable to future users of this system and other systems, as well as to investigators considering development of new programs to extract behavioral parameters from video recordings of *C. elegans*.

2. Materials

1. Dissecting microscope fitted with camera tube and light source (Wild M5A).
2. Digital camera (Sony DFW-V500 Firewire camera).
3. Personal computer with Firewire card and open bay for National Instruments motion controller card.
4. Petri dish positioning system (**Fig. 1**) comprised of:
   a. Four-axis servo/step motion controller (National Instruments, cat. no. PCI-7344).
   b. Four-axis motion wiring connectivity module (controller to power-drive interface; National Instruments, cat. no. UMI-7764).
   c. Interconnecting cable (National Instruments, cat. no. SH68-C68-S).
   d. 4-in. Travel precision square rail miniature linear table (two each) (Daedal Positioning; Parker Automation, cat. no. 402004LN–MPD2L2C4M1).
   e. Base plate (Daedal Positioning; Parker Automation, cat. no. 100-0248-01).
   f. XY adapter plate (Daedal Positioning; Parker Automation, cat. no. 000-9987-01).
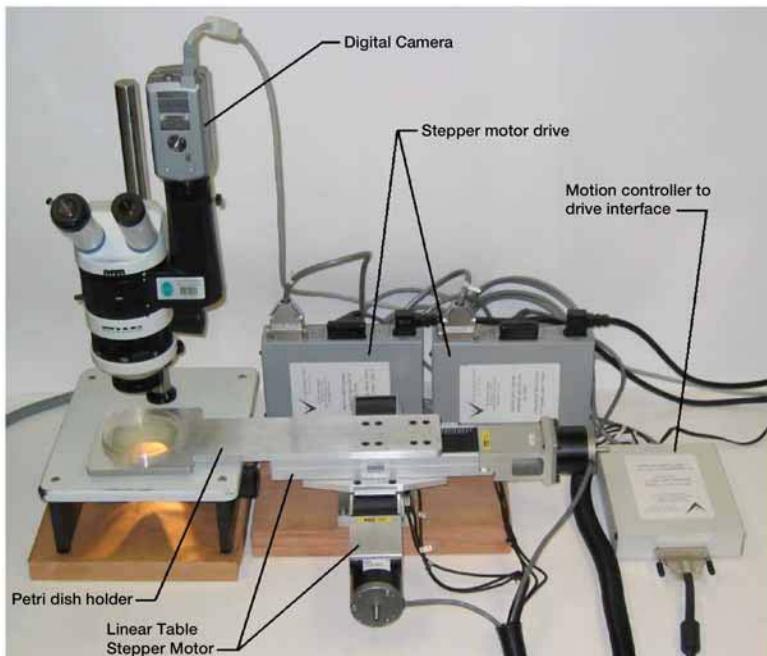
Fig. 1. Tracking system hardware. The digital camera is attached to the tracking system personal computer (PC; not shown) through the PC's Firewire card. The stepper motor for each linear table is attached to its own stepper motor drive. The stepper motor drives are attached to a single "motion controller-to-drive interface," which, in turn, is connected to the motion controller PCI card installed in the PC.

    g. Stepper motor with cable (two each) (Compumotor; Parker Automation, cat. no. ZETA57-51-MO).

    h. Stepper motor microstepping drive (two each) (Compumotor; Parker Automation, cat. no. OEMZL4).

    i. Power supply (5V, 1.2A) (Elpac, cat. no. WM050-1950).

    j. Petri dish holder.

3. Methods

Worm behavior analysis using the combined tracker is a four-step process, corresponding to the four software modules in the system (*see* **Fig. 1**):

1. Capture a subject worm's movement behavior as a video recording (using the Tracker program).
2. Extract and record the worm's time-changing posture and position, abstracting the video-recorded animal as a "backbone" with vertebra-like points distributed along the backbone curve (using the Converter program).

3. Establish and verify the head-tail orientation of the recorded worm's backbone points (using Lineup).
4. Extract and record usable measures of worm behavior and morphology (using Miner).

3.1. Tracker

3.1.1. Prepare for Experiment

The researcher should have worms and Petri dishes prepared for the experiment according to the desired experimental conditions. For example, a typical experimental set-up used at Caltech *(7)* listed in **Subheadings 3.1.1.1.** and **3.1.1.2.**

3.1.1.1. THE DAY BEFORE RECORDING

1. Prepare 5 mL of a fresh OP50 overnight culture and allow it to grow overnight in a 37°C shaker.
2. Place several 100 mm nematode growth medium recording Petri dishes at 20°C to thermally equilibrate for 18–20 h prior to being spread with bacteria.
3. Pick worms at mid-L4 stage onto fresh Petri dishes (spread with a lawn of OP50) 18–20 h prior to beginning recordings to yield a population of age-staged young adult worms for recording. Maintain plates at 20°C until recording begins.

3.1.1.2. THE DAY OF RECORDING

Spread 600 µL of the OP50 overnight culture onto each recording Petri dish. Deposit the solution as a single puddle near the center of the plate then shake, swirl, and tilt the plate to spread the solution evenly across the entire plate surface (should be complete within approx 15 s), finally tilting the plate to one side and drawing off any excess solution that pools within about 5 s. Place the plate in the recording room with its lid off, but covered with a tissue (to prevent dust from accumulating on the recording surface) until the whole recording surface appears dry (the glossy surface attains a matte appearance), which takes 45–60 min. When the recording plates are dry, replace the tissues with the plate lids.

3.1.2. Start Tracker

1. Plug in and power up the whole motion tracking hardware:
   a. The PC.
   b. The microscope light source.
   c. The National Instruments (controller-to-drive interface).
   d. Both Compumotor stepper motor drives.
2. Start the Tracker program by double clicking the Tracker.exe icon. The Tracker user interface will appear on your screen (*see* **Fig. 2**).
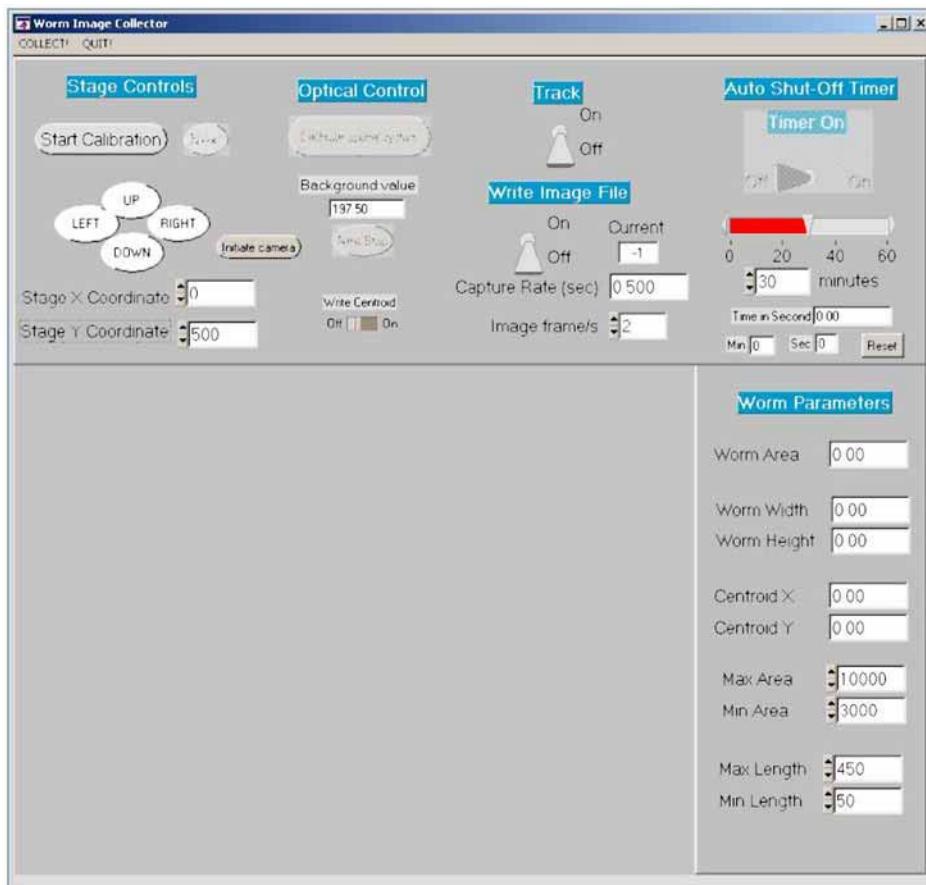
Fig. 2. Tracker user interface. The Petri dish positioning system ("stage") controls are grouped at the upper left of the screen. Recording controls are at the upper right. While tracking, the program displays real-time images of the worm from the digital camera in the field at lower left. The "Worm Parameters" area at the lower right provides controls for defining target ranges for worm area and length, allowing the user to tune Tracker for particular worms of interest.

3.1.3. Calibrate

1. Stage calibration: near the left side of the user interface click the "Initiate Camera" button with your mouse, then click the "Start Calibration" button to adjust the system's automatic stage movements to match the system's camera optics and magnification. Place a Petri dish with nonmoving, identifiable features onto the Petri dish holder. For example, put a mark on the plate lid with a lab marker, or use a plate with eggs or dust—some kind of feature that can be used as a position reference. Follow the instructions in the pop-up windows to move the

reference mark onto the indicated cross-hairs, and if desired for the experiment, define the stage's "home" position.

2. Optical calibration: after completing the stage calibration the optical calibration buttons will be enabled. Place an empty recording plate into the plate holder and click the "Calibrate Optical System" button to set the "Background Value." Follow the instructions in the pop-up windows, adjusting the mirror and light source to achieve a "Background Value" of 170–180, repeating the calibration procedure as necessary. (The current background value is displayed in the readout just beneath the "Calibrate Optical System" button in the user interface.) Note that in the current software release the user interface will not show a live plate image during the optical calibration routine.

3. Frame rate: set the desired target image grab rate using the "Image Frame/s" edit box or use the UP/DOWN arrows. The target frame rate can be set to integer values from 1 to 30 frames per second, but the actual frame rate could be limited by camera or computer capabilities.

4. Auto shut-off: the Tracker program is set up with an automatic shut-off timer to stop recording after a user-specified length of time. The current recording duration is displayed beneath the "Auto Shut-Off Timer" switch on the right side of the user interface. Either the edit box with the up/down arrows or the slider can be used to set the recording duration.

3.1.4. Track

1. Place a subject worm onto a recording plate. Place the recording Petri dish on the Petri dish carrier.

2. Click the "Track" switch to "On" to display a live image of from the recording plate and to begin the worm tracking function. Use the arrow keys on the keyboard or click the "UP," "DOWN," "LEFT," and "RIGHT" buttons on the user interface to move the worm into the user interface video display. With the "Track" switch set to "On" the program should display a black rectangle around the worm in the video display and should repeatedly shift the stage to keep the worm in the camera's view field. If the program does not properly identify the worm with a black rectangle, use the Max and Min Area edit boxes (with UP/DOWN arrows) help define the size range of the worm, and use the Max and Min Length edit boxes to further define the appropriate targets.

3.1.5. Record

1. When the Tracker program is successfully identifying and following the worm on the recording plate, click the "Write Image File" switch to "On." Tracker will display a "Save Image Set File" window to specify the filename for the movie file. Navigate to the desired folder, enter the desired filename, and click "Save." As the program records video of the worm, the user interface displays the moving worm and the elapsed time. When the specified recording time has elapsed Tracker will automatically stop recording and tracking and save the .avi movie of the worm to disk.
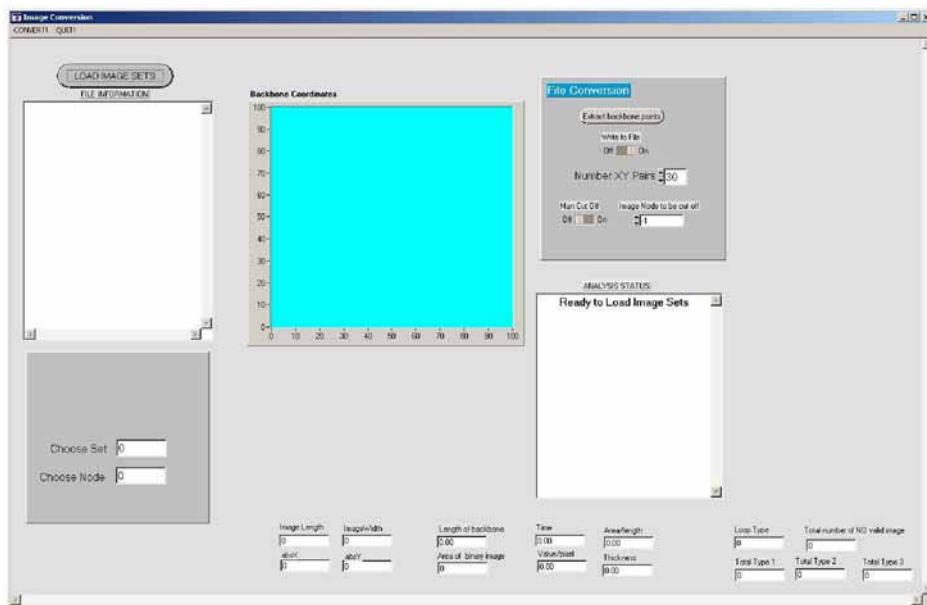
Fig. 3. Converter user interface. The field at upper left displays the list of movie files to be converted and provides the file selection tool. The box at upper right provides the main program controls. During processing, the graph at top center displays the set of backbone points being extracted from the worm movie.

2. Repeat steps 7–9 with all worms. When finished click the "QUIT!" button at the top of the user interface to exit the program.

3.2. Converter

1. Start Converter by double clicking the worm Converter-AVI.exe icon (*see* **Fig. 3**).
2. Click the "LOAD IMAGE SETS" button at the top-left of the user interface. This displays a "Select Files for Viewing and Conversion" window. Navigate to the folder where the .avi files were saved by Tracker, select all of the appropriate movie files, click "Add," then "ok." The selected files will be listed in the "FILE INFORMATION" box on the left side of the user interface.
3. Use the "Number XY Pairs" edit box to select the number of points to be distributed along the worm's "backbone." If in doubt, use the default (30 pairs).
4. Ensure that the "Write to File" switch is set to "On."
5. Click the "Extract backbone points" button at the top right of the display to batch process all of the worm movies.
6. When all of the movies have been processed, Converter will pop up a window indicating "Done." Click "ok" to close the pop-up window and click the "QUIT!"
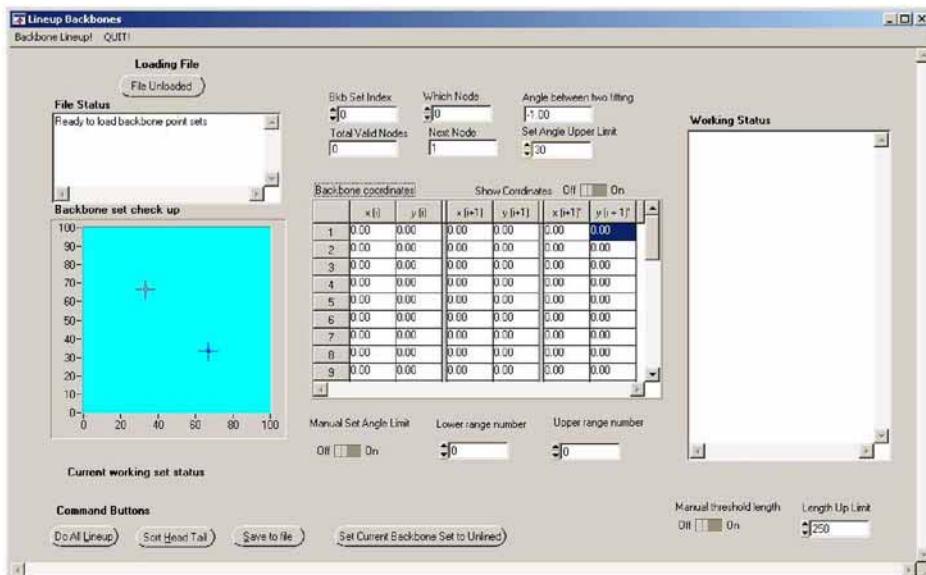
Fig. 4. Lineup user interface. Main program control buttons are along the bottom. A file selection control and display are at upper left. During processing, the graph at left, table at center, and boxes at top center display graphics and statistics about the backbone set being processed.

button at the top of the user interface to exit the program. Converter saves a "backbone" file for each of the movie files, naming them the same as the movie files, except appending "Back.bkb" in place of ".avi."

3.3. Lineup

1. Start Lineup by double clicking the "Lineup.exe" icon (*see* **Fig. 4**).
2. Click the "File Unloaded" button at the upper-left of the user interface to bring up the "Select backbone set for analysis" window. Select the "…Back.bkb" backbone files saved by Converter, click "Add," and then "ok."
3. Click "ok" to close the "Check" window.
4. Click "ok" to close the "Warning!" window.
5. Click the "Do All Lineup" button to line up all of the backbones. Follow any window prompts. When the lineup process is finished click "ok" in the "Warning! The end of the array has been reached" window.
6. Click the "Sort Head Tail" button. In the "Comparison of Speed" window set the "Switch Whole set" switch as appropriate, and then click the "Set It" button. Click "ok" in the "Check It" window.
7. When the "Working Status" box on the right side of the user interface says "All Sets are Sorted" click the "Save to File" button, "ok" in the pop-up window, and
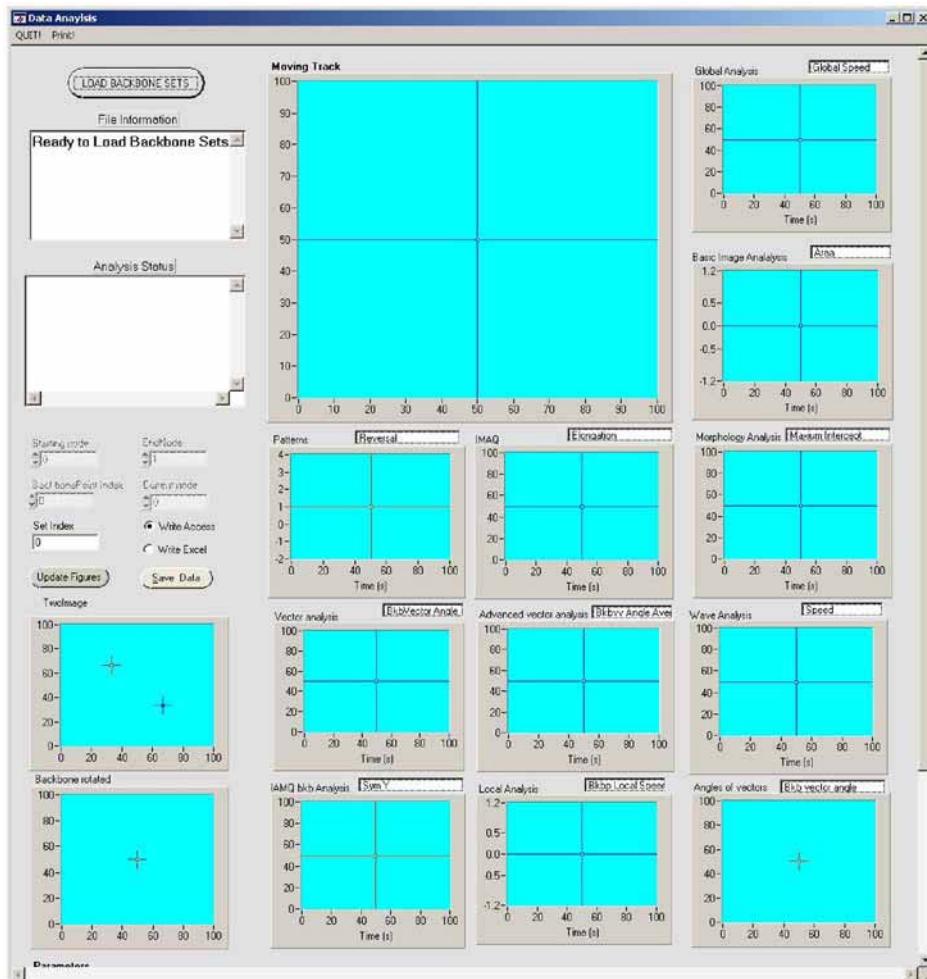
Fig. 5. Miner user interface. The file selection control and status display are at upper left. Charts on the user interface are interactive, allowing the user to explore attributes of interest.

"QUIT!" to exit the program. There will be a new sorted backbone file (.bbs) for each of the (.bkb) unsorted backbone files, saved in the same folder.

3.4. Miner

1. Start Miner by double clicking the "miner_dbg.exe" icon (*see* **Fig. 5**).
2. Click the "LOAD BACKBONE SETS" button. In the "Select Backbone Set for Analysis" window select the desired .bbs sorted backbone files saved by Lineup. Click "Add" and then "ok."

3. Click the "Save Data" button to automatically save all measures of worm behavior and morphology from the loaded .bbs files to Excel files and Access databases, allowing later exploration with commercial scientific software.
4. To use Miner for behavior analysis:
   a. Features for review are grouped by "catalog" (for example, Global Analysis, Wave Analysis, or Angles of Vectors) with a display chart allocated for each catalog. Select a feature of interest from a catalog by clicking on the edit-style box at the top of the chart and using the UP/DOWN arrow keys on the keyboard to scroll through the options.
   b. To explore the range of data for a particular measure, click on the chart for a measure then use the keyboard arrow keys to investigate the data at various time points. The corresponding data for measures from other catalogs at the selected time point are identified by a red crosshair on each chart.
   c. To print and save an interesting chart, activate the chart figure by selecting with the mouse. Then press the "Print!" button on the menu bar.

4. Notes

1. The Petri-dish positioning system described is as assembled and configured by Viewpoint Systems (Rochester, NY; Website: http://www.viewpointusa.com/).
2. The bacteria lawn on the recording plates should be as featureless as possible. An overgrown bacteria lawn on the recording media, holes or scores in the media surface from placing a worm onto the recording plate with a heavy hand, and dust seem to draw worms to themselves and interrupt the worms' normal behavior. Plus, some features can appear like a worm to the machine vision system.
3. Because a bacteria lawn continues to grow during the recording session and the thickness of the food can affect worm behavior, allow only 2–3 h of recording on each set of plates. If a longer recording session is necessary, spread an additional set of plates about 1 h before needed.
4. A high frame rate increases the data resolution, so small changes in behavior should be more perceptible. However, measures of behavior that depend on changes between successive images could show increased noise. For example, worm speed is measured as the change in the worm's position over time; as the frame rate increases the time between successive frames decreases, so any error in the position measurement between successive images will be amplified as data noise in such a calculation.
5. When running Lineup, if there is a question on the proper head/tail orientation of a worm Lineup will pause and ask for human guidance. If necessary, start FrameByFrame by double clicking the "WormConverter.exe" icon in the FrameByFrameAVI folder. FrameByFrame allows the user to access specific images from the worm video by "node" (frame) number or step through the video frame by frame to help identify the proper worm orientation (*see* **Fig. 6**).

   To operate, load the matching .avi file using the "LOAD IMAGE SETS" button. Click the "Display Image Set Nodes" switch to "on." Enter the desired frame number in the "Choose Node" edit box or use the up/down arrows to step forward/backward through the video.
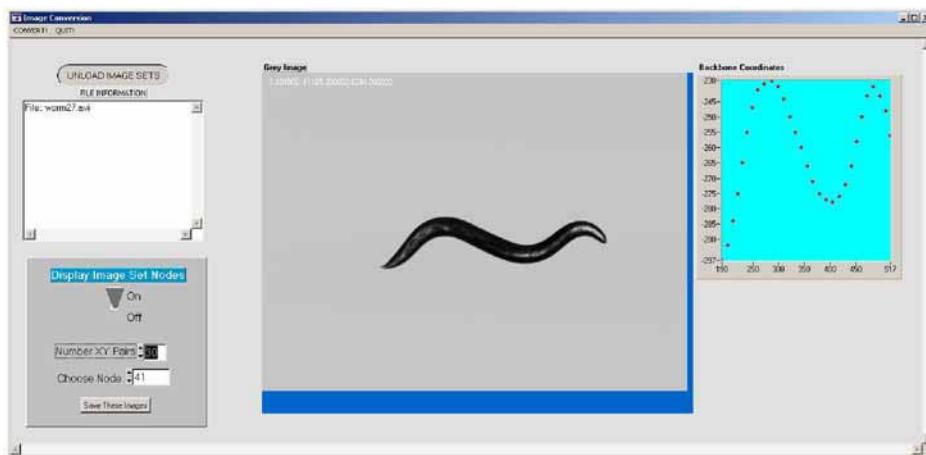
Fig. 6. FrameByFrame user interface. Program controls and file selection tool are at left. The center display and graph at right show images from the worm movie with their corresponding backbone sets.

References

1. Dhawan, R., Dusenbery, D. B., and Williams, P. L. (1999) Comparison of lethality, reproduction, and behavior as toxicological endpoints in the nematode *Caenorhabditis elegans. J. Toxicol. Environ. Health* **58,** 451–462.
2. de Bono, M. and Bargmann, C. I. (2000) Natural variation in a neuropeptide Y receptor homolog modifies social behavior and food response in *C. elegans. Cell* **94,** 679–689.
3. Waggoner, L. E., Zhou, G. T., Schafer, R. W., and Schafer, W. R. (1998) Control of alternative behavioral states by serotonin in *Caenorhabditis elegans. Neuron* **21,** 203–214.
4. Pierce-Shimomura, J. T., Morse, T. M., and Lockery, S. R. (1999) The fundamental role of pirouettes in *Caenorhabditis elegans* chemotaxis. *J. Neurosci.* **19,** 9557–9569.
5. Hardaker, L. A., Singer, E., Kerr, R., Zhou, G., and Schafer, W. R. (2001) Serotonin modulates locomotory behavior and coordinates egg-laying and movement in *Caenorhabditis elegans. J. Neurobiol.* **49,** 303–313.
6. Feng, Z., Cronin, C. J., Wittig, J. H. Jr., Sternberg, P. W., and Schafer, W. R. (2004) An imaging system for standardized quantitative analysis of *C. elegans* behavior. *BMC Bioinformatics* **5,**115.
7. Cronin, C. J., Mendel, J. E., Mukhtar, S., et al. (2005) An automated system for measuring parameters of nematode sinusoidal movement. *BMC Genet.* **6,** 5.

Intracellular Ca²⁺ Imaging in *C. elegans*

Rex A. Kerr and William R. Schafer

Summary

Optical methods provide a noninvasive way to monitor calcium transients in *Caenorhabditis elegans*. Imaging techniques are particularly appealing in *C. elegans* because worms are optically transparent and can be imaged while fully intact. Furthermore, a variety of genetically encoded calcium indicators are available that can be targeted to cells of interest with appropriate tissue-specific promoters. Here, we describe a specific protocol, suitable for monitoring neuronal activity, for rapid calcium imaging in *C. elegans* using the cameleon indicator. Notes are provided to assist with adapting this protocol for use with other indicators and slower time scales.

Key Words: Neuron; muscle; excitable; activity; calcium; imaging; indicator; probe; cameleon; GCaMP; camgaroo; pericam.

1. Introduction

Imaging techniques are widely used to monitor calcium in living cells. Traditional small-molecule calcium dyes have been available for some time, but can be difficult to apply in intact worms owing to the challenges of injecting or otherwise labeling the cells of interest. Fortunately, genetically encoded calcium indicators built from green fluorescent protein (GFP) variants and calcium binding proteins have been developed, allowing precise targeting of the indicator using the wide variety of tissue-specific promoters known in *Caenorhabditis elegans*.

Calcium imaging has been used to monitor the activity and signaling of a variety of cell types in *C. elegans*. Calcium is a key signaling molecule in excitation–contraction coupling in muscles, so calcium is a good indicator of physiologically relevant muscle activity. In *C. elegans*, calcium imaging has been used to study the activity and calcium signaling in the pharyngeal muscle (*1,2*), vulval muscles (*3*), and body wall muscles. Neurons are not guaranteed

to have large calcium transients when electrically active, although voltage-gated calcium channels are commonly found in neurons. However, *C. elegans* does not appear to have voltage-gated sodium channels, suggesting that any active propagation of current in worms occurs via calcium spikes. Calcium imaging has been used as an indicator of neuronal activity in gentle touch neurons *(4)*, in the chemoavoidance neuron ASH *(5,6)*, and the thermosensory neuron AFD *(7)*. Other cell types also use calcium for signaling; for instance, calcium oscillations involved in the defecation cycle have been imaged in the gut *(8)*. Thus, calcium imaging appears to be a broadly applicable method for monitoring cellular activity in *C. elegans*.

A wide variety of calcium indicators are available for optical imaging in vivo, including small-molecule dyes and protein-based indicators. In *C. elegans*, most experiments have been performed with genetically encoded indicators based on GFP variants and the calcium-binding protein calmodulin. The advantage of these indicators is that their expression can be targeted to tissues of interest by expressing the gene encoding the indicator under a cell-specific promoter; this allows highly precise targeting of the calcium indicator with a minimum of invasive preparation required. Unfortunately, these indicators have two primary disadvantages when compared with small molecule dyes: they photobleach more rapidly, thus limiting the duration of imaging experiments, and they have smaller dynamic ranges, which makes calcium transients more difficult to distinguish from noise. However, small molecule dyes have been successfully loaded into nematode cells only by direct injection into gut cells *(8)*, a procedure that is impractical for the tiny (2 μm in diameter) neurons of *C. elegans.* Therefore, we restrict this discussion to genetically encoded indicators, although the principles for measuring small molecule fluorescence are similar.

The genetically encoded calcium indicators come in two major classes. The fluorescence resonance energy transfer (FRET)-based indicators (cameleons) use the conformational change caused by binding of calcium to calmodulin to alter the distance between and relative orientation of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP; **refs. *9–11***). Excitation of CFP yields both direct CFP emission and YFP emission through FRET from CFP to YFP. The degree of energy transfer depends on distance and orientation, so the ratio of YFP emission to CFP emission provides a readout of calcium concentration. Because this indicator is ratiometric, it is relatively robust to motion and changes in illumination intensity. The non-FRET indicators (GCaMP, camgaroo, and pericam) couple conformational changes of calmodulin to the chromophore of a single GFP variant, typically altering its brightness *(12–15)*. These indicators have a greater dynamic range, and some have the advantage that cells with low calcium are dim and therefore do not greatly obscure the

measurement of neighboring cells where calcium is high. Save for ratio pericam *(14)*, these indicators are based on a single intensity measurement, rendering them more prone to artifacts, but simplifying the hardware required. Although the kinetics of these indicators have not been carefully studied, at least one report indicates that fast calcium transients are too brief to detect with the non-FRET indicators *(16)*. We have not performed a detailed comparison between cameleon and other indicators, but have observed calcium transients as brief as tens of milliseconds using cameleon *(4)*. Because both types of indicators have advantages, it is reasonable to try both.

Here, we describe a technique suitable for recording calcium transients in *C. elegans* neurons using the cameleon indicator. This technique should be directly applicable to imaging experiments in other tissues, and with other indicators, over a timescale of seconds to minutes. In particular, we describe methods for sample preparation, for data collection using fluorescence microscopy, and for data analysis.

2. Materials
2.1. Sample Preparation

1. Transgenic worms expressing cameleon in the tissue of interest. The transgenic array does not need to be chromosomally integrated. Preparation of transgenic lines is described in **ref. *17***.
2. Imaging bath solution: 80 m*M* NaCl, 5 m*M* KCl, 20 m*M* D-glucose, 10 m*M* HEPES, 5 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, pH adjusted with NaOH to 7.2. This was modifed from the extracellular saline described in **ref. *18***; the original formulation also works well (*see* **Note 1**).
3. High-melting-point agarose.
4. Heat block at 65°C.
5. Pad-making station. Tape down two microscope slides side by side with just enough room to fit a third slide in between. Add additional layers of tape to define the desired height above the pad.
6. Flat-sided, clear, cold block. A flat-sided Falcon flask filled with water and placed in an ice bucket is suitable.
7. Hair pick. Tape or glue an eyelash to a toothpick or small stick.
8. Nexaband S/C glue (Fisher). Other formulations of glue may also be labeled with the Nexaband brand; the active ingredient in Nexaband S/C is 2-octyl cyanoacrylate (not *n*-butyl cyanoacrylic).
9. Mouth-pressure glue applicator. Take an arm's length of 1/8-in. inner diameter Tygon tubing. Insert a 2-cm length of 1/16-in. inner diameter, 1/16-in. wall thickness Tygon tubing in one end to hold a gluing needle. Insert a clean and sterile 200-μL pipet tip in the other end. Filter tips can prevent unintentional salivation from soiling the tubing.
10. Gluing needles. Pull a standard borosilicate capillary tube leaving an open tip of approx 50 μm using a Flaming/Brown micropipet puller (Sutter Instruments). If

a programmable micropipet puller is unavailable, tips can be broken to size, or the capillary tube can even be pulled by hand.

2.2. Microscopy

1. Upright fluorescence microscope with a ×40 or ×63 water immersion objective.
2. Emission splitter: Dual View (Optical Insights) or W-View (Hamamatsu).
3. Filters: 420/40 CFP excitation filter and 455 excitation dichroic for the microscope; 480/30 CFP emission filter, 505 emission dichroic and 535/30 YFP emission filter for the emission splitter. Suitable filters are available from Chroma and Semrock, among others (*see* **Note 2**).
4. High-speed, low-noise camera, such as the Hamamatsu ORCA ER (or equivalent; *see* **Note 3**).
5. Image acquistion software. MetaVue from Universal Imaging is adequate.

2.3. Data Analysis

1. Image analysis software. We have written custom software to read MetaVue stack files. Image acquisition and analysis software is offered by many vendors and may be suitable (*see* **Note 4**).
2. Data analysis or statistics software. Matlab (The Mathworks) is our tool of choice for serious work, although more graphically oriented software may be easier to learn.

3. Methods
3.1. Sample Preparation

1. Collect worms with sufficiently good expression of the indicator in the tissue of interest. A fluorescence dissecting scope is helpful, but worms can be picked off of any fluorescence microscope with a low-power air objective (*see* **Note 5**).
2. Suck a small amount of glue into the tip of a gluing needle using the glue applicator. The glue will not polymerize rapidly in air, so a single needle can last for hours.
3. Place 60–100 μL of molten 2% agarose in imaging bath solution onto a glass slide in the pad-making station. Place a second slide on top of the drop to create a pad approx 1 mm thick and 8–12 mm in diameter (*see* **Note 6**).
4. Transfer one or more worms to the slide, being careful to minimize the amount of bacteria transferred. Wipe away excess bacteria, as it will prevent good adherence of the glue.
5. Place the slide on the cold block and wait until the worms have stopped moving. Reorient the worms using a hair pick as desired. Overexposure to cold may damage the worms, and excessive condensation will make gluing difficult.
6. Using the glue applicator, place a small drop of glue against the side of the worm's body close to the desired imaging location. Do not cover the worm's nose with glue or swamp the worm in glue; this is likely to kill the worm. Apply additional glue as needed; less glue results in a healthier worm, but worms may wriggle free.

7. Remove the slide from the cold block and blot away any condensation near the base of the pad.

8. Glue the pad to the slide using the glue applicator. Only a small amount of glue is needed to keep the pad from floating off the slide.

9. Cover the pad with a few drops of imaging bath solution to keep the worms from drying out (*see* **Note 7**).

3.2. Microscopy

1. If necessary, adjust the image splitter so that the yellow and cyan channels are side by side and have the same field of view. Sharp edges, grids, and glass needle tips can be useful for alignment. It is usually easier to align at low magnification and with no binning (*see* **Note 8**).

2. Place the slide on the microscope stage and locate the worm. Use additional imaging bath solution to immerse the ×40 or ×63 water immersion objective. If you need to position any additional equipment to conduct the experiment (e.g., a chemical or mechanical stimulation device), do it at this time (*see* **Notes 2** and **9**).

3. Focus on the tissue of interest using transmitted light. If the worm is moving excessively or pulling free from the glue, abandon this worm and move on to the next one. If you have only one worm and it is very important, it may be possible to dry and reglue the worm, but this is not recommended.

4. Refine the focus using fluorescence. Because the indicator will photobleach, it is good to minimize the time and intensity of exposure to fluorescence light. If the tissue of interest is obscured by gut autofluorescence, try to find a worm where there is as little interference as possible.

5. Take a single fluorescence image using the image acquistion software. Adjust the position of the sample, strength of neutral density filters, and exposure time until all of the following conditions are met.

   a. The sample is in the center of the field of view, or at least close enough to prevent it from leaving the field of view if the worm attempts to move.

   b. The field of view contains an indicator-free region. This is necessary for background subtraction.

   c. The camera is not saturated, and will not become saturated when the indicator is activated. For cameleon, this typically involves keeping the brightest pixel in the image at or less than 50% of the maximum that the camera can handle.

   d. The exposure time is fast enough to capture events of interest—at least twice as fast as the fastest event you care about, but 5–10 times faster is preferable. If the timescale of the events is not known, try 100 ms initially.

   e. The illumination intensity is as low as possible while meeting the previously mentioned constraints and leaving the tissue bright enough to collect data that is not too noisy. This typically must be determined with experience.

6. Set the exposure time and number of frames for the desired period of rapid imaging using stream transfer mode. If the stream is too large to acquire conveniently, increase the binning, increase the exposure time, or select a subregion of the full

region to acquire. Note that you may have to readjust the fluorescence illumination intensity to keep the camera from saturating (*see* **Note 10**).

7. Acquire the stream while giving any stimulations necessary for the experiment (*see* **Note 11**).

8. Skim through the acquired data to make sure that the sample stayed sufficiently in focus, did not saturate the camera, and did not photobleach excessively, and save the stream for further analysis if there are no obvious problems. Be particularly aware of any changes caused by the stimulation itself (*see* **Note 12**).

3.3. Data Analysis

Image acquisition software often has data analysis capabilities. The necessary analysis steps are described here, and can be performed by numerous software packages, although doing so may not be fast or convenient. The authors created their own custom software to streamline the data analysis process (*see* **Note 13**).

1. Select a region of the first image in the stream where there is no fluorescence. Compute the average intensity in that region for both the yellow and cyan channels. These are the background fluorescence intensities, Y_{bkg} and C_{bkg}. If the background intensity varies over the course of the recording, you will need to measure the intensity for each image in the stream (*see* **Note 14**).

2. Select a region containing the cell(s) of interest. It is preferable to have a region slightly too large than too small, so that all of the fluorescence emitted from the cell is captured. However, do not let the region overlap with another fluorescent object. This region should cover exactly the same part of the sample on the yellow and cyan channels. If the two regions are misaligned, you may get unexpected results.

3. For each image in the stream, measure the average yellow and cyan fluorescence intensities from the cells of interest, Y_{raw} and C_{raw} and compute the background-subtracted (true) intensites, $Y = Y_{raw} - Y_{bkg}$ and $C = C_{raw} - C_{bkg}$. It may be necessary to move the regions if the sample moves during the stream. Automatic tracking useful in this case. Make sure that the regions stay aligned with each other during tracking.

4. Compute the apparent fluorescence ratio $R_{app} = Y/C$ for each image (*see* **Note 15**).

5. CFP alone emits some light that is picked up through the YFP filters. If you have access to purified CFP or a CFP-expressing sample, you can compute the bleed-through ratio by imaging CFP alone and setting $R_{CFP} = Y/C$. If you do not have access to CFP, $R_{CFP} = 0.6$ is typically a good guess.

6. Compute the fluorescence ratio corrected for CFP bleed-through, $R = R_{app} - R_{CFP}$.

7. Save the list of corrected ratios for further analysis.

8. Using appropriate graphic or analysis software, identify events on the ratio trace $R(t)$. Quantify the size of the event:

$$\Delta R/R = [R(t_2) - R(t_1)]/R(t_1)$$

where t_1 is the time of the start of the event and t_2 is the time of the peak of the event (*see* **Notes 16–18**).

9. Compare events to expected sources of measurement error, such as rapid movement and focus changes, and exclude any questionable data (*see* **Notes 19–22**).

4. Notes

1. A variety of salt solutions are suitable for imaging. We have found that worms survive longer in imaging buffer, but have had reasonable results from other solutions also. If you need to use a different buffer—for example, if you're conducting a chemosensory experiment—it is worthwhile to see how long lightly glued worms stay active and moving on a slide; we typically see worms that can at least be prodded to activity for 4 or 5 h in imaging buffer. Also, keep in mind that altering the buffer may affect the polymerization of the glue.
2. If you are using the filter set described in the materials, keep in mind that there is no emission filter for the eyepieces. It's best not to look through the eyepieces for too long, especially with very bright illumination, as prolonged or repeated exposure to intense blue or violet light can cause retinal damage. Switch to another filter set, such as GFP, while viewing the sample by eye.
3. When selecting a camera, the primary goal is to increase your signal-to-noise ratio. There are three primary sources of noise in charge-coupled device cameras. Shot noise arises from the statistical fluctuations in the number of photons measured and is proportional to the square root of the signal, $F^{1/2}$. Dark current is the leaking of electrons as if there were dim background fluorescence; the leak can be corrected by background subtraction, but the statistical fluctuations cannot and cause noise proportional to the square root of the exposure time, $(\Delta t)^{1/2}$. Readout noise is the inability to accurately count electrons, and typically provides a constant level of noise each time you read a pixel from the camera. Ideally, a camera would minimize all three of these; in practice, because it is impossible to eliminate shot noise, a good camera need only have dark current noise and readout noise that is small compared with the shot noise in your experiments. In particular, for rapid calcium imaging it is usually not critical that a camera have extremely low dark current, because Δt is small. Because the signal-to-shot noise ratio is $F/F^{1/2} = F^{1/2}$, anything that improves the signal decreases shot noise. Cameras can increase the signal by being more sensitive (i.e., having a higher quantum yield), all other things being equal. For example, a camera with quantum yield 0.9 would yield images 50% brighter than those from a camera with quantum yield 0.6, which would improve the signal-to-noise ratio by a factor of $(150\%)^{1/2} \approx 1.22$. Although expensive back-illuminated cameras with quantum yields of 0.9–0.95 are available, the modest increase in signal-to-noise ratio may not be worth the extra cost as compared to interline transfer cameras with quantum yields of 0.4–0.6. Also, there is no benefit in using a camera with higher resolution than you need to resolve your sample, because the greater number of pixels will simply give you greater readout noise. Many cameras have a binning feature that will consolidate pixels before readout (and increase the maximum image capture rate of the camera).
4. The authors express their frustration with the lack of commercially available software that facilitates rapid and correct analysis of fluorescent image sets with moving samples. In particular, achieving proper alignment of the yellow and

cyan channels while tracking moving objects and measuring their intensity with regions of interest is often difficult to manage.

5. We typically observe variable expression levels of cameleon in transgenic worms. This variability is somewhat epigenetic; brightly fluorescing worms tend to have brightly fluorescing offspring. Thus, it can be helpful to pick brightly fluorescing worms for several generations before an imaging experiment.

6. If you need to use a perfusion chamber for your experiment, pads can easily be made on cover slips, or transferred to a cover slip with the aid of a razor blade, and then glued in place. If you are using an inverted microscope, you should make your pads especially thin, because you will be imaging through both the cover slip and the pad.

7. If you do not need to have access to your sample during imaging, you can cover the sample with a cover slip. Be careful to avoid bubbles next to the worm's body; these typically degrade the image quality.

8. If you are using a nonratiometric indicator such as GCaMP, an image splitter is unnecessary. Simply use a standard filter set for the fluorescent protein used by the indicator. Also, keep in mind that intensity-based indicators may get very much brighter in response to calcium influx. Make sure your initial images are sufficiently below the camera's capacity so the camera does not saturate during the response.

9. We recommend water immersion objectives for obtaining high-quality images, especially in an upright scope where there is only solution between the worm and the objective. Imaging through a cover slip with an oil immersion lens is fine also. Air immersion lenses tend to suffer from distortion at the worm's cuticle, although for large structures they are perfectly adequate also. Keep in mind that your sample will dry out while exposed to the air, although larger, thicker pads and high humidity can reduce drying.

10. The protocol here can be used for time-lapse imaging with slight modifications, where separate images are taken at prescribed intervals instead of a single stream. It may be difficult to keep the sample in focus, however. Pads swell slightly over a number of minutes when immersed in imaging buffer, temperature changes can change the relative spacing of the sample and microscope objective, and worms have longer to move, relax, and otherwise change position.

11. If you are providing a stimulus to your worm, it may be difficult to synchronize the stream acquisition and the delivery of the stimulus. To mark the time of a stimulus delivery, one can use a flash of light from an appropriately colored light-emitting diode (LED) at a defined time before the stimulus. A properly located LED will produce a characteristic blip in the background intensity, which can be used as a reference mark for your stimulus. Deliver the flash before the stimulus so it does not disrupt the onset of your stimulus. It is better if you can drive your stimulus from your image acquisition program, or have the image acquisition program set a mark based on an external trigger that is tied to your stimulus, but the LED flash method works well when other options are unavailable.

12. Fluorescence microscopy conditions may change a worm's behavior. In particular, the worms are typically immersed in fluid instead of being in air, which may elicit swimming rather than crawling behavior; being mired in glue is bad for a

worm's health; and exposure to intense fluorescent light (and associated heat) can be noxious. Any behavioral assay required for the imaging experiment should be repeated under microscopy conditions to verify that it still works well enough (although note that some neurons may still be active even if the motor activity of the behavior is suppressed). In particular, we have observed that overly intense fluorescent light inhibits pharyngeal pumping in worms expressing cameleon in the pharynx, and that initial application of fluorescence to worms expressing cameleon in ASH evokes activity in ASH (although later applications do not).

13. Be careful of quantization error. All averages, ratios, and so on, should be performed using floating-point math, and not rounded off to a small number of digits. Rounding will result in peculiar discretized jumps in the ratio, and these jumps introduce extra, unwanted noise into your measurements.

14. The purpose of background subtraction is to attempt to remove light reaching the camera that has a source other than the cell you are interested in. In the case where a cell is over other fluorescent objects (e.g., it is above the gut), simply choosing a nice dark region of the image is insufficient—this will only compensate for general background fluorescence, dark current, and the like, but not the extra fluorescence from the out-of-focus gut granules. If this interference cannot be avoided, choose a ring-shaped region completely outside of but near to the tissue of interest and use this to determine the background. Make sure that the background region is as large as possible while being representative of the background conditions in which the sample finds itself—the background is dim, and therefore noisy. This noise will be added to your measurement noise, so reduce it by averaging as many pixels as possible. In the worst case, your cell may be moving back and forth over objects that create different backgrounds. If this cannot be avoided, you can compensate to some extent by having the background region move along with the object.

15. Note that computing the ratio first, pixel by pixel, and then finding the average ratio over the object is not the same as computing the average intensity and then computing the ratio. The former unfairly weights uninformative and noisy low-intensity pixels. Although ratioing pixel by pixel is useful to produce instructive and colorful images, the ration of average intensities is most appropriate for data analysis.

16. When using a nonratiometric indicator, you have only one channel to monitor and the average fluorescence F (or $\Delta F/F$) becomes the readout of interest. If your illumination intensity is uneven (owing to fluctuations in the lamp, shutter open time if you are opening and closing the shutter for each image, the worm moving between areas of nonuniform intensity in the field of view, and so on), the readout can be corrupted. Some of these factors can be compensated for by having a source of reference fluorescence that does not change (e.g., a fluorescent bead, or a fluorescent protein of another wavelength expressed in the same cell); by measuring this reference value F_{ref} for each frame, you can compute $F = (F_{raw} - F_{bkg})/(F_{ref} - F_{bkg})$ to compensate for some corrupting factors. With a ratiometric indicator, this factor cancels out in the ratio of intensities, so correction is automatic.

17. Measurements of $\Delta R/R$ do not distinguish between long, slowly rising events and fast, rapidly rising events. Thus, it is also useful to measure the duration Δt of

an event (e.g., from start to peak). We have observed mutations that affect only the duration, Δt; only the slope, $(\Delta R/R)/\Delta t$; both in the same direction; and both in opposite directions. Because the duration Δt reflects the duration of the event, while the slope is a measure of the rate of calcium entry, the biological interpretation of the two effects is different.

18. It is not easy to convert from $\Delta R/R$ to calcium concentration. However, with proper background subtraction and correction for CFP bleed-through, $\Delta R/R$ is reproducible between different microscopes and cameras (although it is always best to have a set of validation data to check).

19. FRET-based indicators, such as cameleon, have the property that as one channel gets brighter, the other gets dimmer. Thus, if the sample is sufficiently well behaved and the intensities are stable, an event that causes an increase in $\Delta R/R$ should be accompanied by an increase in Y and a decrease in C. Because many factors can affect the intensity, not every change of $\Delta R/R$ will necessarily be clearly visible in the yellow and cyan channels, but the presence of a reciprocal change helps to verify that a change in fluorescence ratio is caused by a change in calcium influx rather than some artifact.

20. Genetically encoded calcium indicators photobleach when excited by fluorescent light. This process decreases the emitted light from the indicator. In a FRET-based indicator, donor bleaching is unimportant; both channels will get dimmer. However, if the acceptor photobleaches, the ratio will decrease: $R = Y/C$ will be reduced if Y is reduced. Photobleaching can be assessed by placing a sample that should not show activity under fluorescence for an extended period of time and measuring the decrease in R. It is best to avoid significant photobleaching, because by the time significant photobleaching has been achieved, the worm may have been damaged by the fluorescent light and free radical byproducts of fluorescence and photobleaching. If photobleaching cannot be avoided, calculate the bleaching ratio $b = R/R_{initial}$ from the photobleaching trace or from periods of inactivity during your recording. To a first approximation, measured ratio changes $\Delta R/R$ will be too small by a factor of b (*19*). Thus, $\Delta R/bR$ is a less corrupted measurement. Intensity-based indicators typically do not suffer from the same problem; $\Delta F/F$ remains a good measure of intensity change, although biological damage is still a concern.

21. To a first approximation, $\Delta R/R$ (or $\Delta F/F$) reflects the total calcium that has entered a cell. Therefore, turning on a constant calcium current should lead to a linear increase in total calcium, and, therefore, a linear increase in $\Delta R/R$. When the current is turned off, slower calcium removal processes become apparent, and $\Delta R/R$ should relax back to the baseline gradually. Thus, single isolated calcium transients tend to produce a signature change in $\Delta R/R$: an approximately linear rise, followed by an exponential-like decay. Motion and changes in focus can also change $\Delta R/R$, but these rarely occur in this characteristic shape. If the sample undergoes significant motion, it can be useful to plot position or velocity along with $\Delta R/R$ to see if the changes could be explained by motion.

22. Both ratiometric and nonratiometric dyes can produce different results with different expression levels. Because expression levels may be correlated between parents and offspring, and could be different in different strains, it may be diffi-

cult to average away this effect. If the endogenous buffers in the cell have greater capacity than your indicator, then $\Delta R/R$ and $\Delta F/F$ should be the same regardless of the expression level. However, if there as a great excess of indicator, the indicator itself picks up most of the calcium, leaving the excess indicator to sit around uselessly except for creating a bunch of extra background fluorescence. In this extreme, one will observe that $(\Delta R/R)/F$ or ΔF is the same regardless of expression level. Thus, if expression levels vary between mutants and wild-type, or between days when you are running control conditions and running experimental conditions, you should check to see whether the responses $\Delta R/R$ or $\Delta F/F$ are approximately constant with total intensity. We typically find that $\Delta R/R$ is independent of indicator concentration, except in cases of very high expression levels where $\Delta R/R$ is marginally reduced. For this test, note that F should be computed as if there were the same illumination on every sample; if you change the exposure time or use different neutral density filters, scale F accordingly so it more accurately reflects the concentration of fluorescent proteins.

References

1. Kerr, R., Lev-Ram, V., Baird, G., Vincent, P., Tsien, R. Y., and Schafer, W. R. (2000) Optical imaging of calcium transients in neurons and pharyngeal muscle of *C. elegans*. *Neuron* **26,** 583–594.

2. Shimozono, S., Fukano, T., Kimura, K. D., Mori, I., Kirino, Y., and Miyawaki, A. (2004) Slow Ca²⁺ dynamics in pharyngeal muscles in *Caenorhabditis elegans* during fast pumping. *EMBO Rep.* **5,** 521–526.

3. Shyn, S. I., Kerr, R., and Schafer, W. R. (2003) Serotonin and G$_o$ modulate functional states of neurons and muscles controlling *C. elegans* egg-laying behavior. *Curr. Biol.* **13,** 1910–1915.

4. Suzuki, H., Kerr, R., Bianchi, L., et al. (2003) In vivo imaging of *C. elegans* mechanosensory neurons demonstrates a specific role for the MEC-4 channel in the process of gentle touch sensation. *Neuron* **39,** 1005–1017.

5. Hilliard, M. A., Apicella, A. J., Kerr, R., Suzuki, H., Bazzicalupo, P., and Schafer, W. R. (2005) In vivo imaging of *C. elegans* ASH neurons: cellular response and adaptation to chemical repellents. *EMBO J.* **24,** 63–72.

6. Kahn-Kirby, A. H., Dantzker, J. L. M., Apicella, A. J., et al. (2004) Specific polyunsaturated fatty acids drive TRPV-dependent sensory signaling in vivo. *Cell* **119,** 889–900.

7. Kimura, K. D., Miyawaki, A., Matsumoto, K., and Mori, I. (2004) The *C. elegans* thermosensory neuron AFD responds to warming. *Curr. Biol.* **14,** 1291–1295.

8. Dal Santo, P., Logan, M. A., Chisholm, A. D., and Jorgensen, E. M. (1999) The inositol trisphosphate receptor regulates a 50-second behavioral rhythm in *C. elegans*. *Cell* **98,** 757–767.

9. Miyawaki, A., Llopis, J., Heim, R., et al. (1997) Fluorescent indicators for Ca²⁺ based on green fluorescent proteins and calmodulin. *Nature* **388,** 882–887.

10. Miyawaki, A., Griesbeck, O., Heim, R., and Tsien, R. Y. (1999) Dynamic and quantitative Ca²⁺ measurements using improved cameleons. *PNAS* **96,** 2135–2140.

11. Nagai, T., Yamada, Y., Tominaga, T., Ichikawa, I., and Miyawaki, A. (2004) Expanded dynamic range of fluorescent indicators for Ca^{2+} by circularly permuted yellow fluorescent proteins. *PNAS* **101,** 10,554–10,559.

12. Baird, G. S., Zacharias, D. A., and Tsien, R. Y. (1999) Circular permutation and receptor insertion within green fluorescent proteins. *PNAS* **96,** 11,241–11,246.

13. Nakai, J., Ohkura, M., and Imoto, K. (2001) A high signal-to-noise Ca^{2+} probe composed of a single green fluorescent protein. *Nat. Biotechnol.* **19,** 137–141.

14. Nagai, T., Sawano, A., Park, E. S., and Miyawaki, A. (2001) Circularly permuted green fluorescent proteins engineered to sense Ca^{2+}. *PNAS* **98,** 3197–3202.

15. Griesbeck, O., Baird, G. S., Campbell, R. E., Zacharias, D. A., and Tsien, R. Y. (2001) Reducing the environmental sensitivity of yellow fluorescent protein. *J. Biol. Chem.* **276,** 29,188–29,194.

16. Pologruto, T. A., Yasuda, R., and Svoboda, K. (2004) Monitoring neural activity and [Ca^{2+}] with genetically encoded Ca^{2+} indicators. *J. Neurosci.* **24,** 9572–9579.

17. Craig C., Mello, C. C., and Fire, A. (1995) DNA transformation. In: *Methods in Cell Biology, Vol. 48,* Caenorhabditis elegans*: Modern Biological Analysis of an Organism,* (Epstein, E. F. and Shakes, D. C. eds.), Academic Press, San Diego, CA, pp. 451–482.

18. Lockery, S. R. and Goodman, M. B. (1998) Tight-seal whole-cell patch clamping of *C. elegans* neurons. *Meth. Enzymol.* **295,** 201–217.

19. Kerr, R. (2002) Imaging excitable cell activity in *C. elegans*. PhD thesis, Division of Biology, University of California, San Diego, CA.

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In Vitro Culture of *C. elegans* Somatic Cells

Kevin Strange and Rebecca Morrison

Summary

Because of technical hurdles, large-scale cell culture methods have not been widely exploited until recently for the study of *Caenorhabditis elegans*. Culturing differentiated cells from larvae and adult worms is probably not technically feasible because of difficulties in removing the animal's cuticle and dissociating cells. In contrast, large numbers of developing embryo cells can be isolated relatively easily. When placed in culture, embryo cells undergo terminal differentiation within 24 h. Cultured embryo cells have been used recently to characterize ion channel function and regulation and to determine cell specific gene expression patterns. This chapter will provide a detailed description of the methods for isolating and culturing *C. elegans* embryo cells.

Key Words: Cell culture; patch clamp; embryo cells; RNA interference.

1. Introduction

Genetic model organisms provide a number of powerful experimental advantages for defining the genes and genetic pathways involved in biological processes such as Ca^{2+} signaling. The nematode *Caenorhabditis elegans* is a particularly attractive model system (*1,2*). *C. elegans* is well suited for mutagenesis and forward genetic analysis and has a fully sequenced and well-annotated genome. Gene expression in nematodes is relatively easy and economical to manipulate using RNA interference, knockout, and transgenesis. Genomic sequence, as well as many other biological data on this organism, are assembled in readily accessible public databases and numerous reagents including mutant worms strains and cosmid and yeast artificial chromosome clones spanning the genome are freely available through public resources.
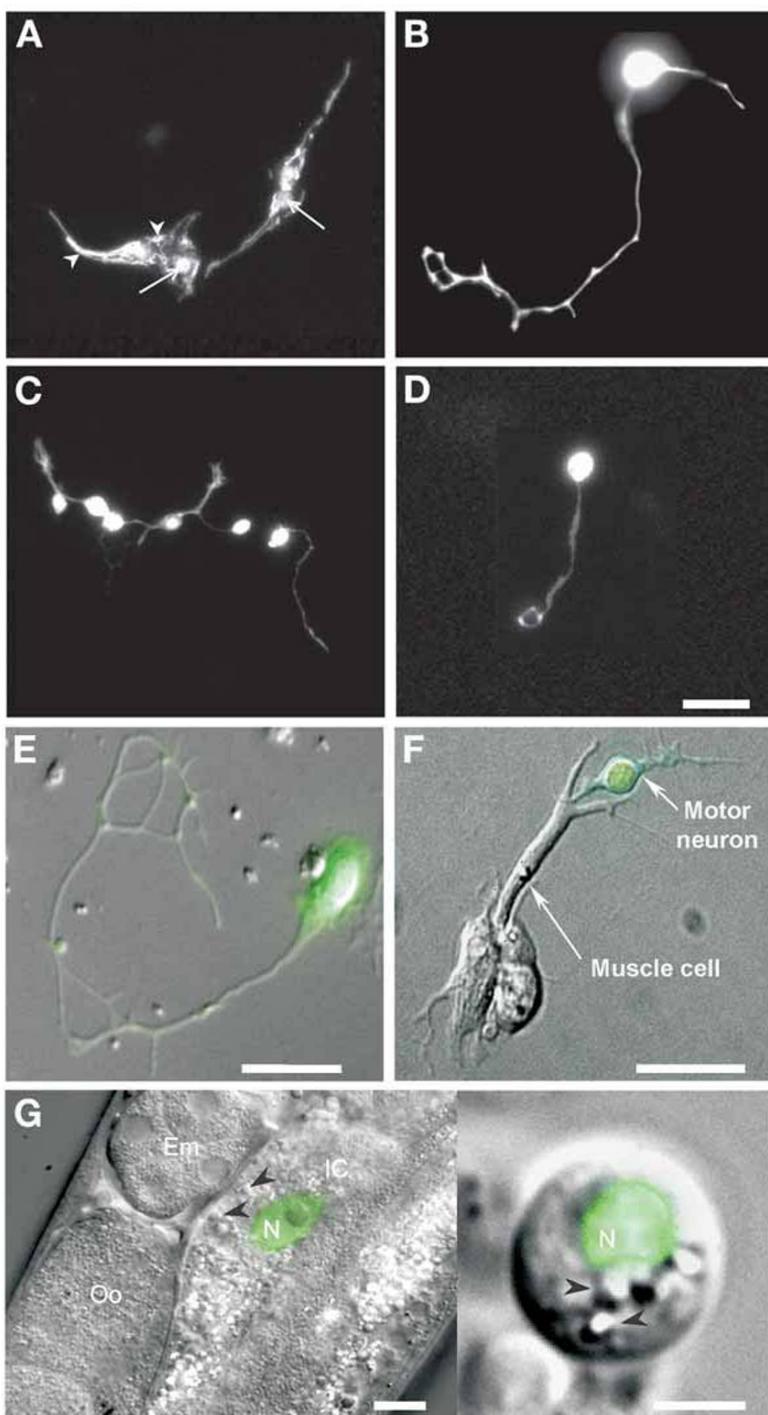
Despite these many experimental advantages, the small size of *C. elegans* and most of its somatic cells, and the presence of a tough, pressurized cuticle surrounding the animal have limited access for molecular studies of cell-spe-

cific gene expression and function. A *C. elegans* cell culture system would provide direct access to individual cell types for functional and molecular analyses. Early attempts at large-scale culture of *C. elegans* embryonic cells were described by Bloom *(3)* and demonstrated the feasibility of culturing differentiated worm neurons. However, Bloom noted significant problems with cell survival, attachment of cells to the growth substrate, cell differentiation and reproducibility of the methods. Initial attempts to patch-clamp-cultured cells were unsuccessful. Buechner et al. *(4)* also reported that cultured *C. elegans* embryonic cells undergo morphological differentiation resembling neurons and muscle cells.

Bloom's studies led to the widely held belief in the field that *C. elegans* cells could not be cultured reliably in vitro. However, using modifications of the method described by Buechner et al. *(4)*, we developed a robust approach for culturing cells isolated from developing worm embryos *(5,6)*. Embryo cells terminally differentiate within 24 h after isolation and in vitro cultures recapitulate the gene expression pattern and cell type frequency seen in L1 larvae *(6)*. In vitro expression of a number of cell-specific green fluorescent protein reporters and molecular markers is similar to that observed in the intact animal (e.g., **Fig. 1**).

Fig. 1. *(Opposite page)* Micrographs of cultured *Caenorhabditis elegans* embryonic cells expressing cell-specific green fluorescent protein (GFP) reporters. **(A)** *myo-3*::GFP expression in cultured body muscle cells. *myo-3* encodes a myosin heavy-chain isoform expressed in body muscles. The *myo-3* reporter strain expresses two GFPs with peptide signals that target them to either the nucleus (arrows) or mitochondria (arrowheads). **(B)** Cultured mechanosensory neuron expressing *mec-4*::GFP. *mec-4* encodes a degenerin-type ion channel subunit expressed largely in neurons that respond to gentle body touch. **(C)** Cultured cholinergic motor neurons expressing *unc-4*::GFP. *unc-4* encodes a transcription factor. **(D)** Cultured neuron expressing *opt-3*::GFP. OPT-3 is a H^+/oligopeptide transporter expressed in glutamatergic neurons. **(E)** Combined differential interference contrast (DIC) and fluorescence micrograph of an *unc-119*::GFP-expressing neuron. *unc-119* encodes a novel protein that is expressed in all neurons. **(F)** Combined DIC and fluorescence micrograph of an *unc-4*::GFP–expressing cholinergic motor neuron physically interacting with a body wall muscle cell. **(G)** Combined DIC and fluorescence micrographs of an *elt-2*::GFP reporter expressing intestinal cell in an intact worm (left panel) and in culture (right panel). Em, developing embryo in uterus; Oo, oocyte in proximal gonad; IC, intestinal cell; N, intestinal cell nucleus. Arrowheads denote refractile granules that are most likely intracellular storage granules. Scale bars in **A–F** are 10 µm. Scale bars in **G** are 10 and 2.5 µm for the whole worm and cultured intestinal cell, respectively. (**A–F** are reprinted from **ref.** *6* with permission of Elsevier Science. **G** reprinted from **ref.** *7* with permission of Rockefeller University Press.)

Cultures can be generated from animals of almost any genetic background. Addition of double-stranded RNA to the culture medium induces dramatic knockdown of targeted gene expression in cultured cells *(6)*. Fluorescence-activated and magnetic-activated cell sorting can be used to enrich cultures for certain cell types. Enriched cultures can in turn be used for cell-specific biochemical, molecular, DNA microarray, and proteomic studies. Cultured *C. elegans* embryo cells have been exploited recently to characterize ion channel function and regulation (e.g., **refs.** *5–14*) and to determine cell specific gene expression patterns *(15–17)*.

2. Materials

2.1. Egg and Cell Isolation

1. Alkaline hypochlorite solution: 1 mL fresh bleach, 0.25 mL 10 N NaOH, 3.75 mL sterile H_2O.
2. Egg buffer: 118 mM NaCl, 48 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 25 mM HEPES, pH 7.3, 340 mOsm.
3. M9 buffer: 42.3 mM Na_2HPO_4, 22 mM KH_2PO_4, 85.6 mM NaCl, 1 mM $MgSO_4$.
4. Chitinase solution: chitinase (Sigma Chemical Co., St. Louis, MO; cat. nos. C6137 or C7809) 1 U/mL sterile egg buffer.

2.2. Culture Vessels

1. For electrophysiology experiments, we culture cells on 12-mm diameter acid-washed glass cover slips. Prior to patch clamping, these cover slips are placed into a bath chamber (model R-26G; Warner Instrument Corp., Hamden, CT) mounted onto the stage of an inverted microscope. A very small bit of vacuum grease is placed in one corner of the bath chamber bottom. This keeps the cell culture cover slip from moving around during solution changes.
2. Depending on the nature of experiments, we also culture cells in 1-, 2-, 4-, or 8-well chambered cover glasses (Nalge/Nunc International, Rochester, NY).
3. For high-resolution differential interference contrast and fluorescence microscopy studies, we usually culture cells in Mat Tek dishes (Mat Tek Corp., Ashland, MA; model no. P35G-0-14-C). These are 35-mm diameter sterile plastic Petri dishes with a no. 0 15-mm diameter glass cover slip glued onto the bottom.

2.3. Cell Culture

1. Peanut lectin solution: peanut lectin (Sigma, cat. no. L0881) 0.5 mg/mL sterile H_2O.
2. L-15 cell culture medium: L-15 cell culture medium (Invitrogen/Life Technologies, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 50 U/mL penicillin and 50 μg/mL streptomycin. Adjust osmolality to 340 mOsm with sucrose and filter-sterilize.

3. Methods

Dissociated embryo cells are prepared using eggs isolated from synchronized cultures of gravid young adult worms. The cell culture protocol thus consists of three major steps:

1. Synchronization of worm cultures.
2. Isolation of eggs.
3. Preparation and culture of dissociated embryo cells.

3.1. Preparation of Peanut Lectin-Coated Culture Vessels

1. Cells must adhere tightly to the growth substrate in order for differentiation to occur. Cells are grown on glass cover slips or in chambered cover slips. The cover slips are coated with peanut lectin to promote cell adhesion.
2. For patch clamp studies, cells are grown on 12 mm diameter acid-washed glass cover slips. Pipet a small volume of the peanut lectin solution onto the cover slip so that it covers about 80% of the surface. When using chambered cover slips, cover the entire surface of the glass, but not the sides of the well.
3. Incubate the cover slips with the lectin solution for 10–20 min and then remove completely. It is important to completely remove the lectin solution. Excess lectin on the cover slips causes cell clumping.
4. Sterilize the cover slips under UV light for several hours. The cover slips can be stored for weeks under sterile conditions. The lectin can also be sterilized by γ irradiation prior to placing it in solution and stored at 4°C (*see* **Note 1**).

3.2. Synchronization of Worm Cultures

1. Isolate eggs as described in **Subheading 3.1.**
2. Resuspend isolated eggs in 100–200 μL of sterile water and seed onto nematode growth medium agar plates without bacteria.
3. Hatch the eggs by incubating the plates at 20–24°C for 12–16 h. Do not incubate for longer than 16 h. With longer incubations worms burrow into the agar, which reduces the final yield.
4. After hatching, rinse the larvae off the plates into a 15-mL conical tube with sterile water or M9 buffer. Pellet the worms by brief centrifugations and then resuspend in a small volume of sterile water.
5. Add washed larvae to 100-mm diameter NA22-seeded, enriched peptone plates. The number of plates needed depends on the yield of hatched worms.
6. Grow larvae to young gravid adults. The time to reach this endpoint varies with temperature, worm strain, and so on. For N2 worms, the incubation temperatures and times listed next work well.

Incubation temperature	Incubation time
24°C	48 h
20°C	64 h
16°C	96 h

3.3. Isolation of Eggs

1. Wash adult worms off agar plates with Milli-Q water into a 15-mL conical tube. Pellet worms by centrifugation at 1200 rpm (~450g) for 3 min in a swinging bucket rotor in a Beckman tabletop centrifuge.

2. Wash pelleted worms with Milli-Q water one to three times until the supernatant is clear of bacteria.

3. After the last wash, lyse the worm pellet with the following mixture of alkaline hypochlorite solution prepared immediately prior to use.

4. Rock worms gently during the lysis. The progress of the lysis reaction is monitored by viewing the worm suspension with a dissecting microscope and the lysis reaction should be stopped when approx 50% of the worms are lysed. Lysis time is critical. Under no conditions should the lysis reaction exceed 5 min. The number of eggs released can be increased somewhat by shaking the tube.

5. Stop the lysis reaction by filling the tube with egg buffer and immediately pellet the eggs and lysed worms by centrifugation at 1200 rpm (~450g) for 3 min.

6. Remove the supernatant using a sterile plastic transfer pipet and wash three times with egg buffer. Make sure the pellet is completely resuspended in the egg buffer during each wash.

7. After the last centrifugation, carefully remove the buffer by sterile plastic transfer pipet.

8. Eggs are separated from debris by centrifugation in a 30% sucrose solution. Resuspend the pelleted eggs and lysed worms in 5 mL of sterile water and then add 5 mL of a sterile 60% sucrose stock. Mix this solution well.

9. Centrifuge the suspension at 1200 rpm (~450g) for 5–6 min using a swinging bucket rotor in a Beckman tabletop centrifuge.

10. Eggs should float in the 30% sucrose solution and will collect at the solution meniscus. Some eggs may appear in the region just below the meniscus. Using a sterile plastic transfer pipet (eggs will stick to glass pipets), transfer the eggs at the meniscus into a sterile 15-mL conical tube. Eggs that stick to the tube can be recovered by gently washing down the sides with a small volume of the sucrose solution and then removing them with a plastic transfer pipet.

11. Collect no more than 3–4 mL of the egg–sucrose suspension. Fill the tube with sterile water and repellet to remove the sucrose.

3.4. Preparation of Dissociated Embryo Cells

1. Isolate eggs from synchronized worm cultures as described in **Subheading 3.3.** After egg isolation, all subsequent steps are carried out in a laminar flow hood under sterile conditions.

2. Remove embryo eggshells by incubating approx 50–200 µL of pelleted eggs in 500 µL of chitinase solution in a sterile Eppendorf tube.

3. Mix the egg suspension in the Eppendorf tube by rocking gently at room temperature for 20–80 min. Note that each lot of chitinase tends to vary in potency.

Consequently, incubation times will have to be assessed for each lot. The progress of eggshell digestion should be monitored by viewing samples of the egg suspension under a microscope.

4. When ≥80% of the eggshells have been lysed, add 800 μL of L-15 cell culture medium to the Eppendorf tube. Pellet the eggs by centrifugation at approx 3200 rpm (~900*g*) for 3 min at 4°C in a microcentrifuge.

5. Carefully remove the supernatant and add 800 μL of fresh L-15 cell culture medium.

6. Gently dissociate the cells by repeatedly pipetting the cell suspension using a 1 mL Eppendorf pipettor. Monitor the degree of dissociation by periodically placing a drop of the suspension on a microscope slide and viewing at ×20. Continue the dissociation until you have a large number of single cells present. The preparation will also contain some undissociated embryos, clumps of cells, and possibly hatched larvae.

7. Pellet the dissociated cell suspension by centrifugation at approx 3200 rpm (~900*g*) and 4°C for 3 min. Remove the supernatant, which may be somewhat cloudy, and resuspend the pellet in 500 μL of L-15 cell culture medium (*see* **Note 2**).

8. The cell suspension is filtered to remove hatched larvae, cell clumps, and very large cells. Filtration is carried out using a 5.0-μ Durapore filter (Millipore Corporation, Bedford, MA) and a 3-mL sterile syringe as follows:

   a. A sterile pipet tip for a 10- to 100-μL Eppendorf pipettor is cut off just below the top "nut." The cut tip is inserted onto a sterile syringe. Alternatively, you can use a sterile 18-gage needle.

   b. 1 mL of L-15 medium is drawn up into the syringe.

   c. The cell suspension is then drawn up slowly into the pipet tip and into the bottom of the syringe. Care is taken so that the cell suspension does not mix with the L-15 medium in the syringe.

   d. The pipet tip is removed and the 5.0-μ filter is placed securely onto the syringe.

   e. The cell suspension is forced through the filter with "medium" pressure into a sterile Eppendorf tube.

   f. To maximize the yield of single cells, the filter is rinsed with L-15 medium as follows. Remove the filter unit from the syringe and attach a new, sterile pipet tip. Draw an additional 1.0–1.5 mL of L-15 medium into the syringe. Remove the tip from the syringe and replace with the filter.

   g. Gently force the L-15 medium through the filter into a second sterile Eppendorf tube.

9. Pellet cells by centrifugation at approx 3200 rpm (~900*g*) and 4°C for 3 min.

10. Remove the supernatants and resuspend the cells in 25–200 μL (the actual volume depends on the size of the cell pellet) of L-15 cell culture medium. After resuspension, pool the two samples.

11. Prepare appropriate dilutions of the cell suspension in L-15 medium and determine cell density using a hemacytometer. Useful counting dilutions range from 1:10 to

1:200. For our experimental purposes, we count only "large" and "medium" cells and ignore any very small cells.

12. Plating density depends on the nature of the experiments that are going to be performed. For patch clamp and optical experiments, we find that a seeding density of approx 230,000 cells/cm^2 is optimal.

13. Plate the cells onto the cover slips in a minimal volume of L-15 medium. Allow the cells to settle and attach for 2 h and then add additional L-15 medium.

14. Culture vessels are kept in small, sealed Tupperware containers lined with wet paper towels. Prevention of medium evaporation is critical. The Tupperware containers are kept in a sterile, humidified incubator at room temperature and ambient air.

15. In our hands, morphological differentiation of cells is largely complete within 24 h. Cell survival is excellent for 2–3 wk. However, these are primary culture and they may dedifferentiate with time. We typically do not use cultures for physiology experiments that are older than 5–8 d.

4. Notes

1. Peanut lectin solutions cannot be filtered sterilized or autoclaved. Ideally, small vials of lectin should be γ irradiated and stored at –30°C until placed into solution. Solutions should be stored at 4°C. Alternatively, irradiation can be performed after coating glass cover slips.

2. Removal of cultured differentiated cells from growth surfaces is difficult and results in cell damage with poor yield. Therefore, in order to enrich cultures for specific cell types, isolated embryo cells expressing cell specific fluorescent reporters such as green fluorescent protein can be sorted by FACS methods *(15–17)* and then plated for culturing.

Acknowledgments

References

1. Barr, M. M. (2003) Super models. *Physiol. Genomics* **13,** 15–24.

2. Strange, K. (2003) From genes to integrative physiology: ion channel and transporter biology in *Caenorhabditis elegans*. *Physiol. Rev.* **83,** 377–415.

3. Bloom, L. (1993) Genetic and molecular analysis of genes required for axon outgrowth in *Caenorhabditis elegans*, Massachusetts Institute of Technology, PhD thesis, Boston, MA, pp. 1–412.

4. Buechner, M., Hall, D. H., Bhatt, H., and Hedgecock, E. M. (1999) Cystic canal mutants in *Caenorhabditis elegans* are defective in the apical membrane domain of the renal (excretory) cell. *Dev. Biol.* **214,** 227–241.

5. Christensen, M. and Strange, K. (2001) Developmental regulation of a novel outwardly rectifying mechanosensitive anion channel in *Caenorhabditis elegans*. *J. Biol. Chem.* **276,** 45,024–45,030.

 6. Christensen, M., Estevez, A. Y., Yin, X. M., et al. (2002) A primary culture system for functional analysis of *C. elegans* neurons and muscle cells. *Neuron* **33,** 503–514.
 7. Estevez, A. Y., Roberts, R. K., and Strange, K. (2003) Identification of store-independent and store-operated Ca^{2+} conductances in *Caenorhabditis elegans* intestinal epithelial cells. *J. Gen. Physiol.* **122,** 207–223.
 8. Yuan, A., Santi, C. M., Wei, A., et al. (2003) The sodium-activated potassium channel is encoded by a member of the *Slo* gene family. *Neuron* **37,** 765–773.
 9. Carvelli, L., McDonald, P. W., Blakely, R. D., and DeFelice, L. J. (2004) Dopamine transporters depolarize neurons by a channel mechanism. *Proc. Natl. Acad. Sci. USA* **101,** 16,046–16,051.
10. Park, K. H., Hernandez, L., Cai, S. Q., Wang, Y., and Sesti, F. (2005) A family of K^+ channel ancillary subunits regulate taste sensitivity in *Caenorhabditis elegans*. *J. Biol. Chem.* **280,** 21,893–21,899.
11. Estevez, A. Y. and Strange, K. (2005) Calcium feedback mechanisms regulate oscillatory activity of a TRP-like Ca^{2+} conductance in *C. elegans* intestinal cells. *J. Physiol.* **567,** 239–251.
12. Bianchi, L., Gerstbrein, B., Frokjaer-Jensen, C., et al. (2004) The neurotoxic MEC-4(d) DEG/ENaC sodium channel conducts calcium: implications for necrosis initiation. *Nat. Neurosci.* **7,** 1337–1344.
13. Suzuki, H., Kerr, R., Bianchi, L., et al. (2003) In vivo imaging of *C. elegans* mechanosensory neurons demonstrates a specific role for the MEC-4 channel in the process of gentle touch sensation. *Neuron* **39,** 1005–1017.
14. Teramoto, T., Lambie, E. J., and Iwasaki, K. (2005) Differential regulation of TRPM channels governs electrolyte homeostasis in the *C. elegans* intestine. *Cell Metab.* **1,** 343–354.
15. Colosimo, M. E., Brown, A., Mukhopadhyay, S., et al. (2004) Identification of thermosensory and olfactory neuron-specific genes via expression profiling of single neuron types. *Curr. Biol.* **14,** 2245–2251.
16. Cinar, H., Keles, S., and Jin, Y. (2005) Expression profiling of GABAergic motor neurons in *Caenorhabditis elegans*. *Curr. Biol.* **15,** 340–346.
17. Fox, R. M., Von Stetina, S. E., Barlow, S. J., et al. (2005) A gene expression fingerprint of *C. elegans* embryonic motor neurons. *BMC Genomics* **6**, 42.

20

Techniques for Analysis, Sorting, and Dispensing of *C. elegans* on the COPAS™ Flow-Sorting System

Rock Pulak

Summary

The COPAS™ Biosorter is a flow cytometer designed to accommodate large objects the size of *Caenorhabditis elegans.* This instrumentation brings high-speed automated analysis and sorting to this small model organism. The Biosort system optically analyzes and sorts living multicellular organisms on the basis of fluorescent protein expression patterns and other optical signatures, at rates up to about 100 organisms per second. The Biosort is capable of fluorescently analyzing and sorting multicellular organisms that are many-fold larger than single cells. Animals pass through a laser beam focused to the center of the flow cell. This beam is narrower than the animal so that multiple measurements are made per animal, which means that the organism is optically scanned along its long axis as it flows. Stable laminar flow in the flow cell acts to orientate the animal with the flow stream. Fluorescent locations along the axis of the animal are sequentially excited as the organism flows through the line of focus. The fluorescent properties of commonly used reagents in the research field allow the user to detect fluorescent protein expression, lectin and antibody binding, and autofluorescence. The ability to dispense organisms as they emerge from the flow cell allows for the collection of those organisms that have certain optical properties defined by the researcher. Also, dispensing allows for the precise distribution of specific numbers of animals for analysis that can vary with organism numbers.

Key Words: *C. elegans*; flow cytometry; dispensing; analysis; size; fluorescence analysis; screening; rare event isolation.

1. Introduction
1.1. COPAS Overview

The COPAS™ Biosort instrument (**Fig. 1**) from Union Biometrica automates the analysis, sorting, and dispensing of *Caenorhabditis elegans* using the physical parameters of animal length, optical density, and the intensity of fluorescent

Fig. 1. Picture of the COPAS™ Biosort with the accompanying desktop computer and associated liquid containers.

markers. Once analyzed, worms can be selected according to user criteria, and then may be dispensed into stationary bulk receptacles or multiwell plates for high throughput screening, dispensing, and collection. If required by the researcher, the analysis and dispensing can be carried out under aseptic conditions. The Biosort instrument can analyze and sort *C. elegans* with about the same precision as manual techniques but with a much higher speed and throughput. By automating the process, the time required for experiments is dramatically reduced, human error is reduced, and new experiments that previously could not be considered are now possible. For example, experiments where large numbers of populations with specific numbers of animals are needed in order to assess a test condition, the Biosort can be used to very accurately set up these populations. Also, instances where numerical data on length or a measurement of fluorescence is required for many individual animals from several populations, the Biosort can collect this data quickly and accurately. Another common application involves the isolation of rare organisms that differ in some optical measurement from a large population of nearly identical animals.

1.2. Sorting Parameters

Five parameters are recorded for each animal as it passes through the flow cell:

1. Optical density of the object of interest (optical extinction).
2. Axial length of the object (size).
3. Green fluorescence (500–520-nm wavelength).
4. Yellow fluorescence (535–555-nm wavelength).
5. Red fluorescence (600–620-nm wavelength).

The Biosort instrument allows for the use of multiple fluorescence excitation and emission wavelengths. In the standard configuration, the instrument has fluorescence detectors for the green, yellow, and/or red regions of the spectrum to cover green fluorescent protein (GFP), yellow fluorescent protein, and DsRed® fluorescent proteins, as well as numerous other commercially available fluorophores.

Sorting rates vary with the concentration of the sample and percentage of the total sample that is being dispensed.

1.3. Technology Platform

Although the COPAS Biosort instrument is designed on the basic principles of flow cytometry, it differs from those of traditional flow cytometers optimized for high-speed cell analysis and sorting in two important areas to permit larger objects to be analyzed:

1. The large-bore fluidics and flow cell design permits handling objects as wide as 150–200 μ.
2. The heart of the COPAS technology is a gentle pneumatic sorting mechanism located after the flow cell that does not harm or change objects, and is, therefore, safe even for the collection of live biological materials or sensitive chemistries.

A constant stream of the worm sample is allowed to flow from a continuously mixing sample cup to a preanalysis chamber, where it is surrounded by a "sheath" solution to produce a stabilized laminar flow. The laminar flow acts to focus the animals to the center of the flow stream and to orient them lengthwise, in the direction of the flow. Animals then pass into the flow cell, where they are illuminated by two low-energy lasers. A red diode laser (670 nm) measures the axial length and the optical density of the object, and a multiline laser is used to excite any fluorophores present. Based on the measured optical parameters (size, optical density, and fluorescence) the operator can then set gated regions for sorting and collecting the population of interest into multiwell microtiter plates or stationary receptacles (**Fig. 2**).

1.4. Software

An IBM compatible computer loaded with the user-friendly COPAS software is provided with every system to provide a dedicated workstation for the analysis, sorting, and dispensing processes. It is worthwhile connecting this computer to your local network to allow for easier data transport or to access the data for further analysis.

Researchers may create, store, and retrieve specific assay files and experimental results using the COPAS software. Optional SFlex software allows the user to deposit different numbers of objects with different sort/gate criteria into each well of a multiwell microtiter plate.
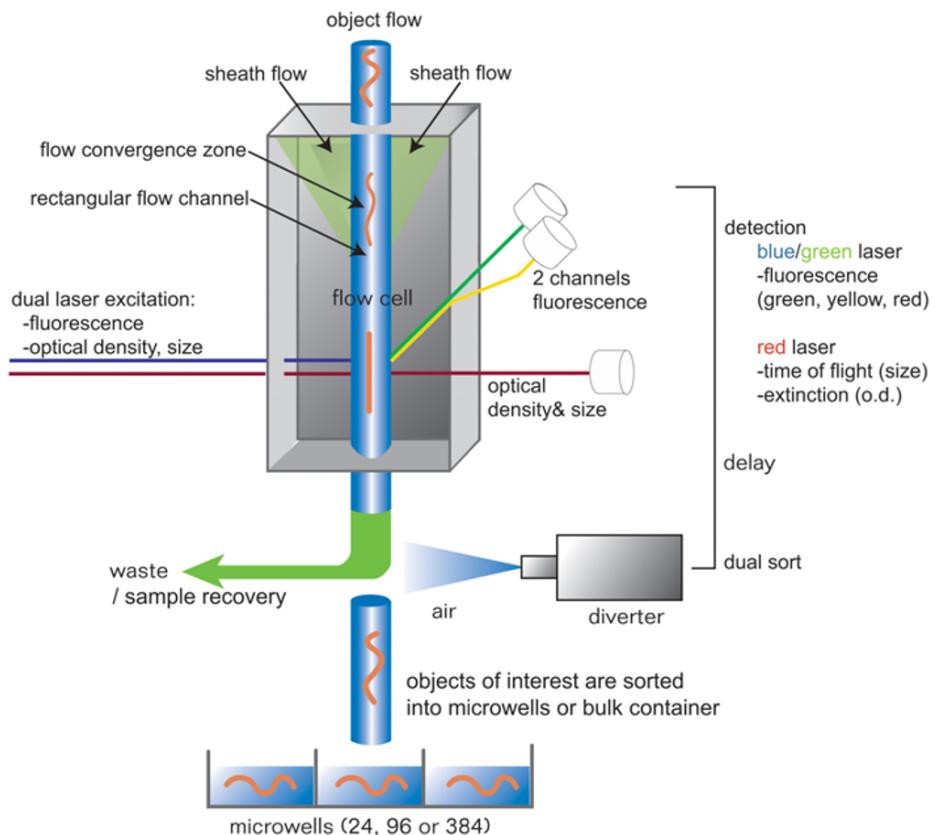
Fig. 2. Schematic of object flow through flow cell showing the path from entry into the flow cell, orientation with the flow because of laminar flow forces, analysis in the flow cell, and sorting event.

1.5. Data Output and Archiving

Raw data collected from the analysis is stored both as a text file and in a format compatible with most flow cytometry software, such as WinMDI. The COPAS software interfaces to common industry analysis tools for further investigation of collected raw data. The numerical raw data can be easily imported into various analysis programs, including commonly used spreadsheet programs to allow for the testing of complex analytical questions and the use of statistics to address subtle biological observations as well as strengthen conclusions from the numerical data.
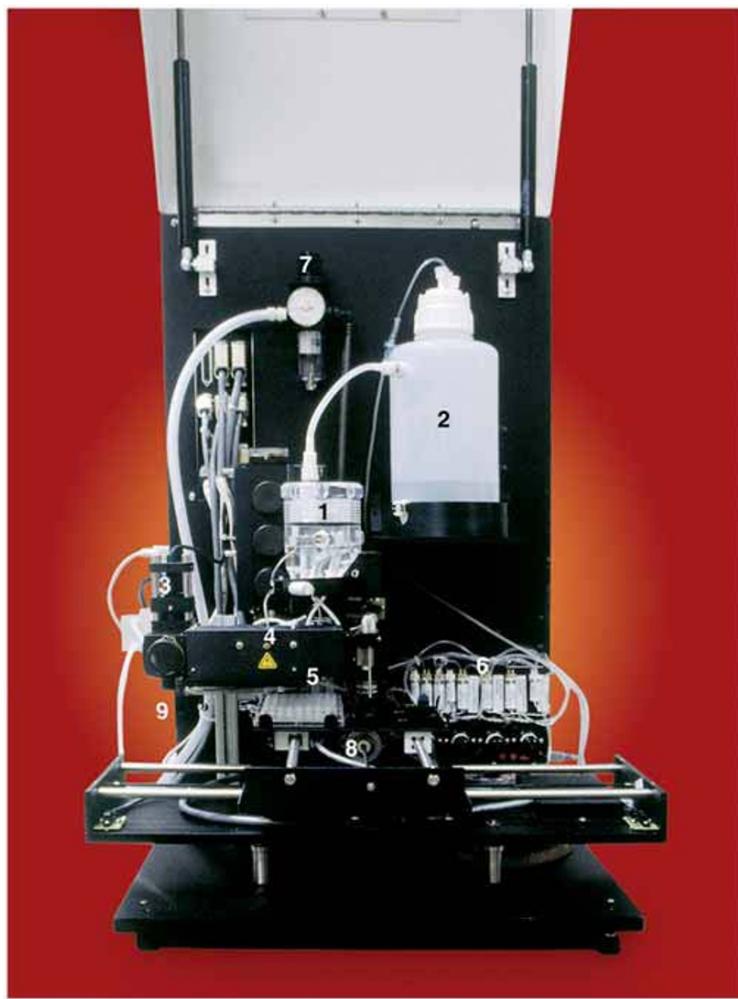
Fig. 3. Internal view of the COPAS system.

2. Materials
2.1. COPAS Biosort Description

Internal views of the COPAS system (**Fig. 3**) are listed below.

1. Primary sample cup.
2. Secondary sample cup.
3. Laser optics assembly.
4. Flow channel.
5. Diverter (sorter) valve.

6. Valve pressure panel.
7. Pressure regulator.
8. Stage.
9. Power switch.

2.2. Description of Biosort Instruments

1. The primary sample cup is located above the optics assembly and can hold approx 40 mL of sample. A full primary sample cup will last for about 45 min of processing. Two stir bars allow continuously mix the sample to prevent sample sedimentation on the bottom. This ensures correct introduction of the sample into the flow cell.

2. Secondary sample container can hold up to 1.5 L of additional sample. The secondary sample cup contains two aerators for continuous mixing. The secondary sample container would only be necessary for certain experiments where very large numbers of animals need to be dispensed or screened. Most experiments can be performed using the 40-mL sample cup.

3. Laser optics assembly consists of a red diode laser and a fiberoptic connection from the external multiline laser focused on a quartz flow channel. A changeable filter used to select excitation wavelengths of 488 or 514 nm is located directly in front of the fiber connection. Make sure the arrow on the filter points away from the laser source. A photon electric sensor is located on the laser beam axis. Orthogonal emitted light is collected by photomultiplier tubes.

4. The flow channel is a quartz assembly containing a 250-μm channel through which the sample flows. The sheath fluid is forced through this channel, producing a stabilized laminar flow condition. The sample is "pushed" or "dropped" into the flow and focused to the center of the flow stream. The laminar flow also orients the objects/organisms lengthwise into a mostly straight configuration.

5. Diverter (sorter) valve sorts the organisms selected by the user. The diverter valve is set to the ON position normally and deflects the fluid stream at about a 35º angle into the waste tray. When an object/organism meeting selection criteria is detected in the flow cell, a message is sent to the diverter valve to turn OFF, thereby permitting the object to drop into a collection device.

6. Valve pressure panel includes four valve regulators. These regulators adjust the pressures for the sheath reagent, the sample, the clean (via back flush), and the diverter. Initial pressures are factory set. Certain instrument and sample conditions may require adjustment of these pressures for sorting efficiency. Also, the user must monitor the pressure and adjust these back to the factory settings if they drift.

7. Pressure regulator allows adjustment and monitoring of the pressure. Pressure may be supplied by an in-house system and is regulated for the system at approx 25 psi.

8. X-Y stage supports the positioning of microtiter plates or other collection devices. Alignment of the stage is required for optimal sample collection.

9. Power switch is located at the rear on the left side of the system.

10. Sheath container is filled with COPAS sheath fluid. A full container of sheath holds 4 L of reagent and lasts for approx 5 h (not shown in picture).

11. Cleanout bottle contains sheath fluid and is used to supply a back flush during the clean cycle. The clean cycle is used to remove air bubbles or any other debris that may have settled during normal system operation that may cause a clog in the flow cell (not shown in picture).

12. External laser is a multiline laser source and is a separate unit. The light is routed to the laser optics assembly by a fiber optic cable. After supplying power to the external laser, it is operated and controlled via the COPAS software (not shown in picture).

2.3. Other Materials

1. Synchronized worms for analysis.
2. Aspirator for quick sample change to remove old sample before adding new sample to sample cup. We typically use a 4-L Erlenmyer flask with a side arm and a vacuum pump or house air.
3. Standard nematode growth medium plates to grow the worms and M9 buffer to wash and collect worms, S Media, and OP50 for growth of animals *(1)*.

3. Methods

3.1. Methods for Fluorescent Measurement of Stress Response

The ability to directly observe the stress response in living animals simplifies the identification of both physical and chemical treatments and genetic alterations that modulate stress response in *C. elegans*. We investigated a stress response in *C. elegans* in a way that lends itself to direct visualization and high-throughput analysis. Various environmental stresses, such as heat, heavy metals, elevated salinity, generate a conserved response in a variety of organisms. Changes in gene expression for a number of genes have been determined and the promoters for these genes can act as sensors for stress. This response has been well characterized in *C. elegans (2–4)*. We have constructed transgenic *C. elegans* expressing reef coral protein ZsYellow and, separately, DsRed2, each under the control of the promoter for the metallothionein gene *mtl-2*. Transgene expression parallels that of the endogenous *mtl-2* gene, and allows direct visualization, localization, and quantification in living animals. We have tested these transgenics for induced expression in response to heavy metal challenge and measured these changes using COPAS instrumentation.

1. Transgenic strains of *C. elegans* that carry a reporter enzyme under control of two different stress-inducible promoters have been created and tested. These reporters allow quantification of subtle responses to certain stresses in intact nematodes. The integrated transgenic strains UB126 (*mtl-2::ZsYellow* + pRF4 [*rol-6 (su1006)*]) and UB274 (*mtl-2::dsRed2* + pRF4 [*rol-6 (su1006)*]) were generated by standard microinjection protocols. Strain CL2070 (*hsp-16-2::GFP, rol-6*) was obtained from the *Caenorhabditis* Genetics Center. The double transgenic strains UB289 (*mtl-2::ZsYellow, hsp-16-2::GFP, rol-6*) and UB290 (*mtl-2::dsRed2, hsp-16-2::GFP, rol-6*) were generated by crossing UB126 and UB274, respectively, with CL2070 and identifying the double transgenics from among the progeny of the double heterozygotes.
2. Synchronized L1 larvae populations of these different single and double transgenic *C. elegans* strains were obtained by a modified procedure of the stan-

Table 1
Threshole, Gains and Scale Recommendations

Mixed Wild-Type Nematode Preparation

Full Scale		Gains			Thresholds	
					Signal	
			Signal	Integral		50
TOF	512					
EXT	512	EXT	80	100		
Green	256	Green	200	200	TOF Minimum	
Yellow	256	Yellow	200	200		10
Red	256	Red	200	200		

PMT Control		
Green	Yellow	Red
900	900	1000

dard alkaline bleaching protocol. The hatchlings were briefly fed to re-enter the life-cycle pathway before the assays were performed.

3. Cadmium dose response assays were performed in 50-mL conical tubes in a final volume of 10 mL in S-basal medium (supplied with *E scherichia coli* OP50) and cadmium concentrations ranging from 0 to 50 μ*M* CdCl$_2$ *(5)*. The samples were incubated on an orbital shaker at room temperature overnight and washed three times with M9 buffer before analysis with the COPAS Biosort.

4. Heat shock assays were performed on seeded nematode growth medium plates. Typically, worms were heat-treated for 3 h at 33°C followed by an overnight incubation at 20°C, washed off the plates with M9 buffer and analyzed.

5. The animals in M9 buffer are added to the 40-mL sample cup and a small sample is run to determine the gating and sorting window. The standard COPAS parameters time of flight (TOF; which is equivalent to length) and extinction (EXT; equivalent to optical density or internal complexity) were used to define a staged population for analysis.

6. Typical instrument settings for early larvae are shown in the **Table 1**. The scale, gains, and photomultiplier tube control levels are values that can be adjusted by the research and the specific values chosen will be depend on the characteristics of the sample being analyzed.

7. We drew a gating region in the TOF vs EXT dot plot to size select the animals of interest from our population. We then acquire data for approx 1000 animals. This data includes the fluorescence intensity of each animal in the sample and their data is stored as both .txt files and .lmd files. The .txt file is raw numerical data for each animal that can be imported into data analysis programs, such as Microsoft® Excel. The .lmd files are formatted for standard flow cytometry software programs, such as WinMDI.

   An example of part of the .txt file is given next. Each row represents the values for a single organism. For example, the animal with a TOF of 430 has an EXT value of 491, a green fluorescence value (FLU1) of 2036, and a red fluorescence value (FLU2) of 2037. Other data from the .txt file has been excluded.

TOF	EXT	FLU1	FLU2
430	491	2036	2037
200	216	1868	1720
298	429	2037	2036
96	135	886	806
370	283	2037	2037
340	309	2036	2036
354	333	2037	2036
265	230	572	734
320	270	104	195
11	8	70	103
277	291	2036	2035
333	385	578	797
302	382	685	829
307	305	2037	2037
260	342	2037	2035
342	361	2037	2036
326	358	2037	2036
248	277	2035	2035
320	392	2036	2037

8. The collected data from the COPAS Biosort were imported into WinMDI for analysis. A region in the TOF vs EXT dot plot was defined that was similar to the region originally defined in the COPAS Biosort software to analyze the sample. Mean values of TOF, EXT, and the corresponding fluorescent colors within this region were estimated and the quotients mean green fluorescence/mean TOF (and, if applicable, of the second fluorescent protein [mean yellow or red fluorescence/mean TOF]) were determined and graphically displayed in Microsoft Excel. This method of analysis showed that these strains do respond to heat shock and to cadmium stimulation with gene expression from the respective promoters. The final more detailed analysis of this data will be presented elsewhere.
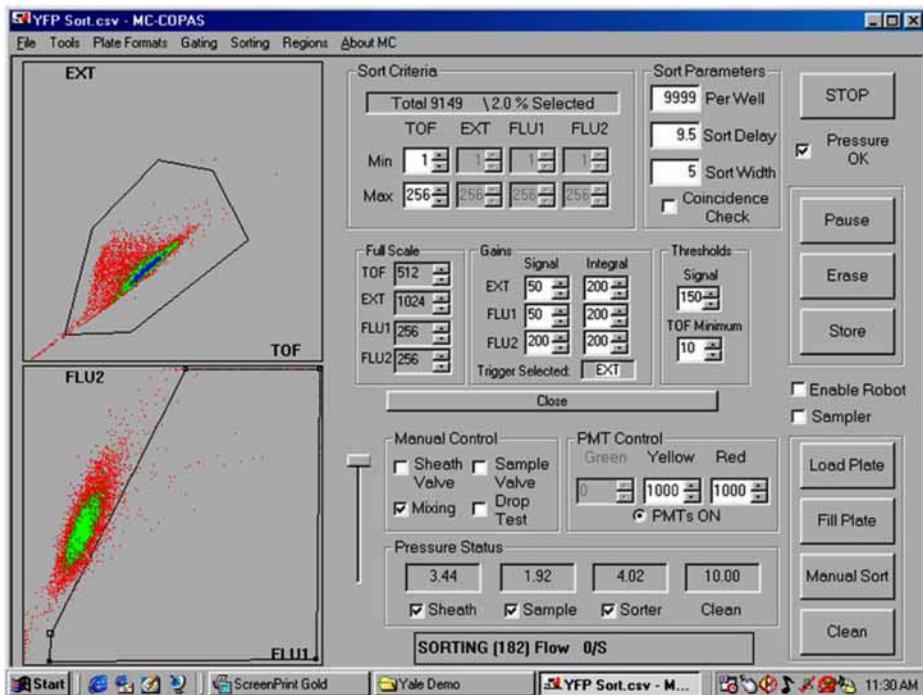
Fig. 4. Screen captures of COPAS software main screen DOT-PLOT view with gate region selected (time of flight vs extension, upper left side) and the Sort Region selected (FLU1 vs FLU2, lower left panel). The Sort Region includes organisms that have increase yellow fluorescence (ZsYellow expression) relative to the red auto-fluorescence. The majority of the organisms are not expressing the yellow fluorescent protein from this uninduced UB289 strain but a small fraction of organisms are.

3.2. Methods for Sorting Rare Events

Gene expression from a transgenic strain can vary in unexpected ways. For example, we see that the regulated metallothionein gene mtl-2 promoter will occasionally express even in the apparent absence of inducer in the transgenic strain UB126. Whether this observation mimics a natural misfiring of the endogenous regulation or if it is an effect of the transgenic construct is not certain but using the COPAS Biosort, we are able to sort and dispense these rare events for further analysis. This is a rare event and induction can be mild, with low expression from the promoter (**Fig. 4**).

Similar approaches have been used to look for misexpression of a transgenic marker in the opposite direction. For example, a screen of a mutagenized population using a constitutive tissue-specific promoter such as str-1::GFP (PY1089,

kindly provided by Piali Sengupta and Cory Bargmann) can yield animals that express more or less of the fluorescent reporter. This type of screen is very difficult to do at a dissecting microscope. Finding a slight increase in fluorescence in a background of weakly fluorescencing animals is a difficult task. Likewise, finding a dimmer animal in a population of fluorescing animals is also a challenge. However, such rare events can be identified and isolated using the Biosort.

1. L4 larvae of the integrated *str-1::GFP* strain, PY1089, were mutagenized with ethyl methanesulfonate by a standard method, transferred to eight separate Petri dishes and allowed to grow. F_2 progeny where synchronized from each plate and represent one pool of mutagenized animals and were analyzed and sorted using the Biosort. A total of 16 different pools were analyzed.
2. The standard COPAS parameters TOF and EXT were used to choose a tightly staged population for analysis. These where analyzed for fluorescence levels and animals with lower levels and with higher levels were sequentially sorted from these F_2 pools. The candidate mutants were sorted one per well into 96-well plates. Mutants were confirmed by running them through the Biosort a second time and visually by microscopy.
3. Approximately 50,000 animals were screened and 1000 were dispensed to wells in the first round. One hundred-eighty four lines from these sorted animals were retested. Not all of these passed secondary screening. From certain pools we recovered multiple isolates with the same pattern of expression. These cannot be unambiguously considered as independent isolates and, therefore, our collection probably represents approx 13 independent mutations.
4. The collected data from the Biosort were stored as text files and imported into Microsoft Excel for analysis. We determined the fluorescence for each organism and combined these data with their corresponding EXT values from the Biosort. EXT vs FLU DOT-PLOT images were generated, the slope of each sample was determined and compared with unmutagenized PY1089 animals. This provides a numerical representation for each particular strain.

    This application defines a general method for finding both *cis*-acting mutations that affect the expression of the transgene and potential transacting factors that are involved in regulating the transgene expression.

References

1. Sulston, J. E. and Hodgkin, J. A. (1988) Methods. In: *The Nematode* Caenorhabditis elegans, (W. B. Wood, ed.), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 587–606.
2. Braeckman, B. P., Houthoofd, K., and Vanfleteren, J. R. (2001) Insulin-like signaling, metabolism, stress resistance and aging in *Caenorhabditis elegans*. *Mech. Ageing. Dev.* **31,** 673–693.
3. Johnson, T. E., Cypser, J., de Castro, E., et al. (2000) Gerontogenes mediate health and longevity in nematodes through increasing resistance to environmental toxins and stressors. *Exp. Gerontol.* **35,** 687–694.

4. Lithgow, G. J. (2000) Stress response and aging in *Caenorhabditis elegans*. *Results Probl. Cell Differ.* **29,** 131–148.

5. Cioci, L. K., Qiu, L., and Freedman, J. H. (2000) Transgenic strains of the nematode *Caenorhabditis elegans* as biomonitors of metal contamination. *Environ. Toxicol. Chem.* **19,** 2122–2129.

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