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Using *Caenorhabditis elegans* to Study Serpinopathies

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Abstract

Protein misfolding, polymerization, and/or aggregation are hallmarks of serpinopathies and many other human genetic disorders including Alzheimer's, Huntington's, and Parkinson's disease. While higher organism models have helped shape our understanding of these diseases, simpler model systems, like *Caenorhabditis elegans*, offer great versatility for elucidating complex genetic mechanisms underlying these diseases. Moreover, recent advances in automated high-throughput methodologies have promoted *C. elegans* as a useful tool for drug discovery. In this chapter, we describe how one could model serpinopathies in *C. elegans* and how one could exploit this model to identify small molecule compounds that can be developed into effective therapeutic drugs.

1. Introduction

A serpinopathy is a conformational disease in which a genetic mutation predisposes a serpin protein to misfold and polymerize. The accumulation of abnormal proteins within cells lead to tissue injury and degenerative disease (Belorgey *et al.*, 2007; Gooptu *et al.*, 2009; Lomas and Carrell, 2002; Silverman *et al.*, 2007). Examples include α 1-antitrypsin (AT) deficiency, hereditary angioedema, familial encephalopathy with neuroserpin inclusion bodies (FENIB), and thrombophilia. Mutations occur most commonly in or near the hinges of the reactive site loop and the shutter region that underlies the opening of the β -sheet A. One of the best-studied serpinopathies is AT deficiency. AT deficiency is most commonly caused by a mutation that results in a substitution of the amino acid, glutamic acid at position 392 to a lysine (known as the Z mutation). This change in charge, destabilizes the hinge region making the AT protein more prone to polymerization. These protein polymers are poorly secreted and accumulate within the endoplasmic reticulum (ER) of liver cells (Perlmutter, 2002). Since AT is the predominant serine peptidase inhibitor in extracellular fluids, decreased AT secretion results in a loss-of-function phenotype manifest by peptidase-inhibitor imbalance, connective tissue matrix destruction, and susceptibility to chronic obstructive lung disease. In contrast, the accumulation of aggregation-prone α 1-antitrypsin Z (ATZ) in liver cells leads to a gain-of-toxic-function phenotype characterized by liver failure and carcinogenesis (Rudnick and Perlmutter, 2005). Indeed, AT deficiency is the most common genetic cause of pediatric liver disease and the most frequent genetic diagnostic indication for liver transplantation during childhood. Curiously, only ~10% of all

ATZ homozygous individuals develop severe liver disease (Sveger, 1988). This observation suggests that other genetic and/or environmental factors play key roles in determining disease severity and outcome. Our current understanding of these “other” factors is rudimentary and effective therapeutic options are very limited.

Model systems have provided valuable insights into various human diseases. For example, transgenic worms and fruit flies have enhanced our understanding of Alzheimer’s, Parkinson’s, Huntington’s, Duchenne muscular dystrophy, diabetes, cancer, and a plethora of other complex diseases. Thus, simple model systems may be useful for unraveling previously undiscovered information about serpinopathies and provide insights into the cellular mechanisms that protect against protein misfolding.

Caenorhabditis elegans is a well-established model organism with a proven track record. Their small size (~1.5 mm as an adult), short life-cycle (2–3 days), high fecundity (~300 progeny in ~3 days), easy genetic manipulation, and ease of culture, makes them an excellent, low-cost, alternative to other model organisms. Transgenic animals can be generated with relative ease and well-established reverse and forward genetic tools are readily available. Recently, techniques for large-scale liquid worm culture, high-throughput worm sorting, and automated high-content imaging have been described (Gosai *et al.*, 2010), paving the road for the use of *C. elegans* in drug discovery.

A comprehensive description of basic *C. elegans* methodology is beyond scope of this chapter. We refer interested readers to *C. elegans: A Practical Approach* (Stiernagle, 1999), for further information. This chapter will focus on methods relating to the study of serpinopathies in *C. elegans* with particular emphasis given to the generation of transgenic animals and high-content drug screening.

2. Considerations for Transgenesis

Transgenic animal approaches can provide a wealth of information regarding how a gene is regulated and what function it performs within the context of a whole organism. In a null mutant background, altered (mutated) copies of a gene can be easily reintroduced to study structure–function relationships. In some cases where a serpin null fails to yield an overt phenotype, overexpression of a gene may provide insights into protein function by perturbing the serpin–proteinase balance (Pak *et al.*, 2004). Transgenes fused to one or more fluorescent proteins (FPs) or affinity tags can be exploited for the study of protein–protein interactions and target protein identification (refer Chapter 14 for details on using *C. elegans* to identify *in vivo* serpin targets). Transgenic worms can also be used to study toxicology and as a tool for drug discovery.

A wide array of *C. elegans* strains, plasmids, promoters, reporter genes, and RNAi reagents are readily available thanks to the generosity of the *C. elegans* research community (Fire *et al.*, 1990). A list of useful *C. elegans* resources and their websites is shown in Table 13.1.

2.1. Fluorescent Protein (FP) fusions

Before attempting to generate a model of a serpinopathy in *C. elegans*, a number of important factors must be carefully considered. For example, if the transgenic line will be used to study protein synthesis and disposition, an FP tag may be helpful for visualization of the transgene (Chalfie *et al.*, 1994). FP tags obviate the need for fixation and antibody staining, and allows one to monitor proteins in real time in live animals. In addition, FP tags are useful in screens looking for genetic mutations or small molecules that alter misfolded protein accumulation. Alternatively, if the transgenic animals will be used to study protein–protein interactions, it may be useful to add one or more affinity tags to aid detection and purification of protein complexes (Hobert and Loria, 2005).

2.2. DNA origin

The source of DNA that will be used to generate the transgenic animal is another important consideration. If a *C. elegans* homologue can be readily identified, the endogenous gene can be used to minimize complications arising from cross-species differences in the coding region. If a clear worm homologue cannot be readily identified, human DNA may be used, however, modifications may be required to ensure efficient expression. Due to variations in RNA splicing and the large size of most human genes it is often not possible to directly express human genomic DNA in worms. However, transgene expression in *C. elegans* is enhanced by the presence of intronic sequences (Okkema *et al.*, 1993). As such, it is often necessary to clone several synthetic introns into human cDNA sequences to ensure efficient transgene expression.

2.3. Promoter choice

The promoter used to drive expression of the transgene is another important consideration. Transgene expression should be directed in cells or tissues that most closely resemble the biological environment in which the human protein is normally expressed. A large number of *C. elegans* promoters with various spatial and temporal specificities have been characterized. A few examples are listed in Table 13.2.

2.4. *C. elegans* expression vectors

A wide variety of modular *C. elegans* expression vectors have been generated and made available to the *C. elegans* research community by Fire *et al.* (1990). A schematic of the canonical expression vector is shown in Fig. 13.1. All expression plasmids contain an *Escherichia coli* ampicillin resistance gene, multiple cloning sites, introns, and a 3'UTR (Fig. 13.1A). Numerous vectors with built-in tissue-specific promoters, various fluorescent reporter proteins with nuclear localization, secretion, and specific organelle (e.g., mitochondria) targeting signals are also available. A typical expression vector used to generate transgenic animals expressing an aggregation-prone serpin (such as ATZ) fused to GFP is shown in Fig. 13.1B.

2.5. Transgenesis methods

The most widely used method for transgenesis in *C. elegans* is microinjection. DNA is injected into the gonads of a young adult hermaphrodite (Mello and Fire, 1995). DNA is

then taken up by the germ cells and transgene expression is seen in developing worms within 24–48 h. Transgenes are typically injected as plasmids, however, PCR products can also be used obviating the need for cloning (Hobert, 2002). Injected DNA forms large extrachromosomal arrays that are passed on to subsequent generations in a non-Mendelian inheritance pattern (Stinchcomb *et al.*, 1985). Transmission rates can vary anywhere from 5% to 95%, so multiple independent transgenic lines should be isolated.

Microparticle bombardment is an alternate method for *C. elegans* transgenesis. DNA is bound to small microbeads (usually gold particles) and ballistically bombarded into worms. Bombardment is commonly used to generate animals carrying low copy numbers of the transgene. This is a particularly important consideration if one is concerned that an overexpression of a transgene might be toxic to the animal. In some cases, stable integrated transgenic lines can be obtained (Praitis *et al.*, 2001). However, bombardment is more expensive, labor intensive, and time-consuming than microinjection.

2.6. Coinjection markers

If the transgene of interest is not readily identifiable (i.e., does not have a fluorescent tag or is anticipated to be expressed at low levels), it may be necessary to use a coinjection marker to facilitate selection of positive transgenic lines following injection. Coinjection markers typically recombine with other DNA in the mix and are incorporated concomitantly into the extrachromosomal array. A coinjection marker can therefore be used as an indirect indicator of transgenesis. Coinjection markers often make use of a promoter that is specific to an anatomical structure remote from the region of interest and is tagged with an FP of a different color to reduce interference during multicolor imaging. If a fluorescent microscope is not available, one may utilize a coinjection marker that induces a clear dominant phenotypic change or rescues a mutant phenotype. For example, *rol-6* induces a unique “rolling” movement and transgenic animals can be easily identified under a standard dissecting microscope. Some commonly used coinjection markers are described in Table 13.3.

A flow chart of the transgenesis procedure is illustrated in Fig. 13.2. In brief, the serpin gene of interest is cloned into a *C. elegans* expression vector under the control of an appropriate promoter (Fig. 13.2A). The plasmid DNA (along with a suitable coinjection marker) is microinjected into the gonad of a young adult hermaphrodite (Fig. 13.2B). Positive transgenic lines are identified and propagated (Fig. 13.2C). Stable integrated lines are generated by exposure to ionizing radiation (Fig. 13.2D). Stable integrants are outcrossed to remove nonspecific mutations that may have been introduced by exposure to radiation.

3. Microinjection

3.1. Microinjection equipment and reagents

- *Injection table*: A heavy marble table or air table to minimize vibrations.
- *Inverted DIC microscope*: Zeiss Axiovert 100 or equivalent instrument, equipped with standard 5× and 40× objectives. The microscope should have a flat, free-sliding glide stage to allow for easy manipulation of slides.

- *Micromanipulator*: Narishige MMN-1 or equivalent. Microinjections require a manipulator that can hold and position a needle with fine mobility in the *X* (forward and back), *Y* (side to side), and *Z* (vertical) planes and allows for easy adjustments of needle angle and location.
- *Pressurized injection system*: FemtoJet (Eppendorf; Hauppauge, NY catalog # 920010504) is an injection needle system in which the needle is placed in a holder with a tight seal collar, which is attached by plastic tubing to a regulated pressure source.
- Standard dissecting microscope.
- *Dehydrated agarose pads*: Provide a “sticky” surface for immobilizing worms during microinjection.
- *Microinjection needles*: Femtotips (Eppendorf, catalog # 930000035) are a high-quality injection needle.
- *Injection oil*: Halocarbon Oil 700 (Sigma; St. Louis, MO catalog # H8898).
- *Recovery buffer*: Phosphate buffered saline (PBS).

3.1.1. Agarose pads

1. A drop (~100 μ l) of hot 2% agarose (dissolved in H₂O) is placed onto a 24 $\times$ 100 mm microscope glass coverslip (avoid creating air bubbles).
2. A second coverslip is immediately placed on top of the agarose.
3. When the agarose has hardened (~10 s), the top cover slip is gently removed.
4. Steps 1–3 is repeated until desired number of agarose pads are made.
5. Agarose pads are placed onto a tray, covered with aluminum foil and allowed to dry for a period of 1–3 days.
6. Agarose pads are stored in the original glass coverslip box for many months.

3.2. Preparation of plasmid DNA for injections

1. *E. coli* clones harboring the appropriate expression vectors are grown overnight.
2. Plasmid DNA is purified using the Qiagen (Valencia, CA) mini prep kit (or similar).
3. DNA injection solution: 10 μ l final volume.
 - a. Transgene plasmid final concentration ~50–75 ng/ μ l (if gene is toxic, the plasmid concentration can be lowered).
 - b. Coinjection marker final concentration ~5–30 ng/ μ l.
 - c. Total DNA concentration ~80 ng/ μ l (Bluescript plasmid DNA is added as required to adjust the final DNA concentration).

3.3. Microinjection method

1. The injection mix is centrifuged for 10 min at 13,000 rpm to pellet particulate matter that may clog the needle.
2. 2 μ l of DNA solution is carefully placed on the top of the injection needle and allowed to fill the tip of the needle by capillary action.
3. The micromanipulator is then depressurized to allow mounting of the loaded injection needle.
4. Once the needle is mounted, a worm is placed on an agarose pad and covered with a drop of halocarbon oil to prevent desiccation.
5. Using the 5 $\times$ objective, the position of the worm is adjusted to ensure correct orientation for injection. The dorsal surface of the worm should be at an $\sim$ 45–75 $^{\circ}$ angle to the needle (see Fig. 13.3(1)).
6. Upon focusing on the worm, coarse controls on the micromanipulator are used to position the needle near the gonad of the worm.
7. Using the 40 $\times$ objective, final adjustments are made to ensure that the gonad and the tip of the injection needle are in the correct focal plane.
8. The needle is then gently inserted into the cytoplasmic core of the gonad (see Fig. 13.3(2)).
9. DNA is then injected by applying short bursts of pressure (1300 psi; see Fig. 13.3(3)).
10. If performed correctly, DNA should move freely through the gonad syncytium without disrupting any germ cell precursors (see Fig. 13.3(4)).
11. Immediately following injections, the agarose pad carrying the injected animal is transferred to the dissecting microscope.
12. The injected worm is recovered using a small drop (20 μ l) of PBS buffer.
13. Once the worm is freely moving about, it is transferred to a fresh Nematode Growth Medium (NGM) agar plate. [NGM: 3.0 g NaCl, 2.5 g Bacto-peptone, 20.0 g Agar dissolved in 1.0 l of double-distilled H₂O; solution is autoclaved and then cooled to 55 $^{\circ}$ C to which the following solutions are added: 1.0 ml cholesterol (5 mg/ml in Ethanol), 1.0 ml 1 M CaCl₂, 1.0 ml 1 M MgSO₄, 25.0 ml 1 M KH₂PO₄, pH 6.00] (Hope, 1999).

Steps 6–13 should be performed as quickly as possible to prevent desiccation of the worm. To ensure sufficient transgenic animals are generated, at least 5–10 worms should be injected.

3.4. Isolation and propagation of transgenic worms

1. An injected worm is placed on a 60-mm NGM plate seeded with a lawn of *E. coli* OP50 strain. This is repeated for each injected worm. (Alternatively, two to three

injected worms may be placed on one 60-mm seeded plate if one is injecting eight or more worms.)

2. The injected worm is allowed to recover and is examined daily for progeny expressing the desired transgene or coinjection marker.
3. Expressing progeny in the F1 generation are segregated to separate plates and propagated as distinct lines. It is recommended that as many expressers as possible in the F1 generation be selected and propagated as it is common for expression to be reduced or lost in the F2 generation of some strains.
4. Any subsequent progeny are transferred to a new seeded 60-mm plate and the progeny observed for continued transmission of the transgene or coinjection marker.
5. After approximately three generations, the expression and transmission of the transgene is relatively predictable and stable.

3.5. Stable integration of transgenes

Once relatively stable lines of transgenic animals have been isolated, they can be either maintained as extrachromosomal arrays or integrated into the genome depending on the intended use. Extrachromosomal arrays are generally simple to maintain but require constant diligence to ensure that animals expressing the desired transgene are propagated under the desired conditions. For example, if the transgene transmits a survival disadvantage compared with unaffected progeny, then care must be taken to not allow conditions of stress (starvation, temperature fluctuation, etc.) to affect the animals. This can be time-consuming and, for that reason, one might consider integrating the transgene.

Integration involves exposure of the transgenic animals containing extra-chromosomal arrays to ionizing radiation that causes double-stranded DNA breaks (Mello *et al.*, 1991). Upon repair of these breaks, the extrachromosomal array may be incorporated into a chromosome of the animal. The radiation may also cause unintended mutations in other genes, so outcrossing following isolation of stable integrated lines is required (Evans, 2006). Integration of extrachromosomal arrays requires a significant investment of time and resources but can result in a more stable and predictable transgenic line. When selecting lines to integrate, keep in mind that lines with a generally low transmission rate (less than 30% of progeny) will be significantly easier to integrate. In the end, the distinguishing feature of the integrated population is that it will transmit the transgene to 100% of progeny. This distinction will be easier to identify in lines with a low transmission rate to begin with.

During the integration procedure, lines are allowed to starve several times. This facilitates integration in a couple of ways. First, it allows for several generations to pass following irradiation and may help remove lethal spurious mutations introduced by the radiation. Second, it imparts a competitive advantage to transgenic animals with a high transmission rate. After starvation, recovered transgenic animals are more likely to have higher transmission rates.

Prior to beginning, prepare the following (per line to be integrated):

- 33 × 100 mm NGM plates seeded with OP50.
 - 30 × 60 mm NGM plates seeded with OP50.
 - 210 × 35 mm NGM plates seeded with OP50.
1. 10 transgenic adult worms are transferred to 2 × 100 mm NGM plates and allowed to grow for 2–3 days until there are maximum progeny at the late L4 stage. (For a more closely synchronized population, adults may be allowed to lay eggs for several hours, then removed and the eggs allowed to develop to late L4.)
 2. On the day of irradiation, 200 late L4 stage transgenic worms are selected and placed on a plate.
 3. Worms are exposed to 3500 rads (35 Gy) of radiation.
 4. The irradiated worms are allowed to recover for several hours at room temperature (or overnight at 16 °C).
 5. Two worms are transferred to one 100-mm plate. A total of 30 plates are inoculated.
 6. These plates are incubated to starvation at room temperature (7–10 days).
 7. The worms are washed off with 5 ml of PBS and transferred in 50- μ l aliquots to a new 60-mm plate. This is repeated for each of the 30 plates to new 60-mm plates.
 8. These plates are incubated to starvation at room temperature (4–5 days).
 9. The worms are washed off with 1 ml of PBS and transferred to new 35-mm plates in 25- μ l aliquots. Repeat for each of the 30 plates to new 35-mm plates.
 10. The worms are allowed to develop for 2 days.
 11. From each of the 30 plates, six transgenic worms are selected and placed one each on a new 35-mm plate ($6 \times 30 = 180$ total plates).
 12. The worms are allowed to grow for 3 days at room temperature.
 13. The plates are examined for 100% transmission.
 14. The integrated strains are outcrossed several times to remove spurious background mutations.

We recommend that several integrated lines be isolated and stored. Transgene integration sites are determined by SNP mapping. A description of mapping techniques is beyond the scope of this chapter. Readers interested in these techniques should refer to *C. elegans*, a practical approach (Hope, 1999) or www.wormbook.org for more information.

3.6. Characterizing new transgenic lines

Once a new transgenic line has been established, regardless of whether it is by extrachromosomal array or integrated, one can characterize the phenotype of that line. Basic studies include assays for postembryonic development (PED), brood size, and longevity. These techniques are described in detail in the following chapter. If one is using an FP, then

thorough imaging should also be undertaken. One of the advantages of *C. elegans* is that it is a transparent multicellular organism and is very amenable to high-powered imaging. One can utilize coinjection with fluorescent markers to colocalize FPs to subcellular structures such as lysosomes or endoplasmic reticula.

4. High-Content Drug Screening

High-content screening (HCS) has been developed as a tool to expand mainstream drug development for cells in multiwell plates (Giuliano *et al.*, 2006; Gosai *et al.*, 2010; Haney *et al.*, 2006; Johnston and Johnston, 2002; Johnston *et al.*, 2007, 2008, 2009; Nickischer *et al.*, 2006; Trask *et al.*, 2006; Williams *et al.*, 2006). While unicellular models are extensively used for drug discovery, they lack phenotypic diversity and multiorgan complexity of whole organism models (Bleicher *et al.*, 2003; Hodgson, 2001). Thus, HCS campaigns utilizing a whole organism model can offer information regarding drug absorption, distribution, metabolism, excretion, or toxicity (ADMET) at the earliest stages of screening (Gleeson, 2008; Gleeson *et al.*, 2009) and improve chances of identifying efficacious drugs (Gosai *et al.*, 2010).

4.1. Equipment, consumables, and reagents

- VPrep (Velocity11; Menlo Park, CA): Automated liquid handling device for transferring liquids across microtiter plates.
- Flex Drop dispenser (PerkinElmer; Waltham, MA): Automated liquid dispenser for microtiter plates.
- COPAS BIOSORT (Union Biometrica; Holliston, MA): Large particle (animal) sorter and dispenser.
- Cellomics Arrayscan VTI (Thermo Scientific; Pittsburgh, PA): Automated fluorescence imaging and analysis platform.
- Optical Bottom plates (Nalge Nunc International; Rochester, NY. 164730).
- Compound Library (Sigma-Aldrich; St. Louis, MO. LO1280-1KT).
- *E. coli* (OP50 strain).
- 10-cm seeded NGM plates.

4.2. OP50 food preparation for drug screening

1. A single OP50 colony is used to inoculate 3 ml of sterile LB.
2. Culture is incubated at 37 °C with vigorous shaking overnight.
3. The following morning, 1 ml of the culture is used to inoculate 1-l sterile LB broth prepared in a 2-l flask.
4. One liter culture is then incubated at 37 °C with vigorous shaking until OD₆₀₀ = 0.5. This usually takes 2–4 h.

5. Bacteria is separated into 4×250 ml centrifuge tubes and pelleted by centrifugation at $4500 \times g$ for 5 min.
6. Pellet is washed twice with 200 ml PBS and a small aliquot is removed to measure OD_{600} .
7. Volume of PBS is adjusted accordingly so that a 1:100 dilution of the bacteria suspension has an $OD_{600} = 1.0$.
8. Unused bacteria suspension can be stored at -80°C indefinitely by adding equal volume of 50% glycerol.
9. When ready to use, bacteria is thawed and pelleted by centrifugation for 10 min at $4500 \times g$.
10. Supernatant is removed and pellet is resuspended in half the original volume using PBS.

4.3. Compound library preparation

A diverse set of compound libraries are available from a number of academic and commercial sources (e.g., National Institutes of Health (NIH), Chem-Bridge, MayBridge, Prestwick, ChemDiv, and Sigma-Aldrich). When developing a screening method, it is important to assess the quality of the assay using a manageable compound library. The Library of Pharmacologically Active Compounds (LOPAC) is a modestly sized library considered to be a gold standard in assay validation. It consists of 1280 small molecule modulators that affect a diverse range of cellular processes and represents most major drug target classes. Compounds libraries are supplied in 96- or 384-well formats. Libraries are frequently prepared using DMSO as the primary solvent.

1. Compounds library is replicated from the master plate by transferring 2 μl of each compound to daughter plates using the VPrep liquid handling device. Space should be left of the plates to provide treatment free controls.
2. Plates are sealed with aluminum plate seals and stored at -80°C .
3. When it is time to prepare assay plates, daughter plates are thawed at 37°C and centrifuged at $50 \times g$ for 60 s.
4. Seals are removed and Flex Drop dispenser is used to add 98 μl of S-medium to each well.
5. Fifteen microliter of diluted compounds are then transferred to four sets of optical bottom 384-well assay plates (Nalge Nunc International; Rochester, NY).
6. Assay plates are then covered with aluminum plate seals and placed at -80°C for long-term storage.
7. On the day of sorting, assay plates are thawed at 37°C and centrifuged at $214 \times g$ for 60 s.
8. Fifteen microliters of fresh $4 \times$ assay medium (4.0 ml OP50, 25.4 ml sterile S-medium, 0.6 ml antibiotic antimycotic solution (100 $\times$ stabilized BioReagent,

sterile-filtered, with 10,000 units penicillin, 10 mg streptomycin, and 25 μ g amphotericin B per milliliter. Sigma-Aldrich, A5955), 120 μ l 100 mM (+)-5-fluoro-2'-deoxyuridine (FUDR) to prevent growth of progeny) is added to each well.

4.4. Animal preparation and sorting using the COPAS BIOSORT

To minimize variability in the assay, it is imperative to start a drug screen with a tightly synchronous population of worms expressing an uniform level of transgene. Although, animal preparation methods may vary slightly depending on the model and the specific assay being employed, we recommend using late L4–young adult stage worms. For a high-throughput drug screen, worm transfer via traditional hand-picking would not be a viable option. As such, we utilized a flow cytometer specifically designed for worms and other multicellular organisms called COPAS BIOSORT. This is a high-throughput, pressure driven system that has the capacity to perform multiparametric analysis and sorting. A screenshot of the COPAS BIOSORT interface is shown in Fig. 13.4. In this example, worms are initially gated to isolate a desired developmental stage using empirically determined time-of-flight (TOF) and coefficient of extinction (EXT) values. Animals are then sorted into assay plates based on GFP fluorescence intensity and TOF.

1. 6–12 transgenic L4 stage animals are picked by hand onto 12 10-cm NGM plates.
2. Plates are incubated at a permissive temperature for 1 week or until the F2 generation reaches adulthood.
3. Gravid adults are isolated using the COPAS BIOSORT to select highest GFP expression (~30% of the population).
4. 12,000 sorted animals are transferred to 10-cm seeded NGM plates, ~ 1000 animals per plate.
5. Animals are allowed to lay eggs for 5–8 h.
6. Adults are removed by gently washing plates with 2 ml of PBS. Eggs will remain stuck to the bacteria on the plates.
7. Eggs are hatched by 16-h incubation at a permissive temperature.
8. Hatchlings are washed off and transferred to ~100 seeded NGM plates. More or less, plates might be used depending on the total population size. Care must be taken to prevent starvation or overcrowding.
9. Animals are incubated for 36 h or until most of the animals on the plates are in the L4-young adult stages.
10. Worms are transferred to 50-ml conical tubes by PBS wash.
11. Animals are allowed to pellet by gravity for 5 min and supernatant is discarded.
12. S-medium (minus EDTA; 11.7 g NaCl; 2 g K₂HPO₄; 12 g KH₂PO₄; 10 mg cholesterol; 20 ml 1 M potassium citrate; 20 ml trace metal solution: [0.346 g FeSO₄·7H₂O; 0.098 g MnCl₂·4H₂O; 0.144 g ZnSO₄·7H₂O; 0.012 g CuSO₄·5H₂O; 500 ml H₂O, filter sterilize and shield from light]; 6 ml 1 M CaCl₂; 6 ml MgSO₄;

200 μ l Triton X-100 in 2 l H₂O. filter sterilize) is used to adjust the worm suspension to a concentration of 400 animals per milliliter.

13. Prior to sorting, sheath and cleanout bottles are filled with filter-sterilized S-medium containing 0.01% Triton X-100.
14. Animals suspended in S-medium are added to the sample cup in 40-ml aliquots.
15. Flow rate of the BIOSORT is adjusted up to 25 worms per second to prevent the discarding of desired samples and coincidence check is enabled to enhance specificity.
16. 35 animals are sorted into the wells of the 384-well assay plate (containing drugs and OP50).
17. Sorted plates are sealed with ThinSeal (ISC Bioexpress; Kaysville, UT. T-2417-4) and incubated at room temperature for 24–48 h.

A starting population of ~45,000 animals is necessary to dispense 35 worms into every well of a 384-well plate given a 30% selection rate. This is to offset any worms that were discarded during the sorting process due to the coincidence check. Coincidence check detects when the BIOSORT cannot separate a desired and undesired animal flowing through the system and will discard both.

4.5. Automatic image acquisition

Images are acquired with the ArrayScan V^{TI} fitted with a 2.5 $\times$ objective and a 0.63 $\times$ coupler. ArrayScan V^{TI} is a high-content screening instrument designed for high-capacity automated fluorescence imaging. Quantitative data analysis is performed using the SpotDetector BioApplication optimized to identify FP accumulation in *C. elegans*. Transgenic animals expressing an aggregation-prone serpin fused to GFP in the intestine and mCherry in the pharynx are generated for the drug screen (Fig. 13.5). A two-channel (mCherry and GFP) assay is used to acquire images of these transgenic worms. Images acquired in the GFP channel are converted to numerical data using complex algorithms to assess the extent of misfolded protein accumulation defined as “spots.” Parameters that can be measured include total spot count, total spot area, and total spot intensity. Image acquisition and analysis of a 384-well plate is rapid and takes less than 45 min to complete. Data is subjected to sophisticated statistical analyses to identify hit compounds. In this example, a hit compound is defined as a compound that significantly reduces the total GFP spot area or intensity. Using this approach, we recently identified compounds that significantly reduced accumulation of misfolded ATZ in *C. elegans* (Gosai *et al.*, 2010).

1. 30 μ l of 20 mM NaN₃ is added to each well to anesthetize worms.
2. Plate is resealed and mixed by inversion.
3. Plate is incubated for 7 min at room temperature to ensure all worms are anesthetized.
4. Seal is removed and plate is imaged using the ArrayScan V^{TI}.

5. Using Protocol Interactive, the exposure time of each channel is set to 25% saturation using the no treatment controls.
6. Sample wells are then selected and imaged using the Scan Plate function.
7. After the scan is finished the data is exported to excel for further analysis.

4.6. Considerations for HCS

It is important to understand the goals of the screen from the earliest stages of preparation. Transgenic animals that will maximize the assay quality must be isolated and chromosomal integration is essential for screening efficiency. Further, it is also important to optimize incubation temperature, assay duration, and developmental stage as transgene expression and response varies heavily with these parameters. Optimization and assessment of a potential high-throughput screening (HTS) assay is accomplished by performing a series of experiments using a large number of positive and negative control samples ($n = 100$). Assay quality is quantified using Z' -factor analysis. The Z' -factor of an assay can be calculated using the equation: $Z' = 1 - ((3 \times (\sigma_p + \sigma_n)) / (\mu_p - \mu_n))$ where σ is the standard deviation, μ is the mean and p and n are positive and negative controls, respectively. A Z' -factor between 1.0 and 0.5 indicates an excellent assay, between 0.5 and 0.0 indicates a good assay, and below 0.0 indicates a poor assay.

The screen should be performed in duplicate to increase selectivity. This will ensure that compounds with the most reproducible effects will be identified. Initial hits are subjected to secondary assays to confirm results. To validate the efficacy of a potential hit, dose response and time response experiments are performed. Compounds that provide promising dose- and time-dependent responses are then chosen for further validation in cell culture and mouse models.

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Figure 13.1.

A schematic representation of a *C. elegans* expression plasmid. (A) The canonical vector used to build all expression vectors (Okkema et al., 1993). (B) An expression vector for expression of an aggregation-prone serpin protein in *C. elegans*. I, synthetic intron; UTR, untranslated region.

Figure 13.2.

Transgenesis flow chart. (A) Plasmid DNA for injection are prepared. (B) DNA is injected into young adult hermaphrodites. (C) Transgenic progeny are isolated and enriched. (D) Stably integrated transgenic lines are established by exposure to irradiation.

Figure 13.3.
Schematic of microinjection into *C. elegans* gonad.

Figure 13.4.

Automated worm transfer using COPAS BIOSORT. A screenshot of the COPAS BIOSORT interface is shown. Dots in the scatter plots represent an individual animal. Animals are gated based on their TOF and EXT values which are parameters for size and granularity, respectively (*polygon, upper* scatter plot). Animals are further gated based on their GFP expression (*polygon, lower* scatter plot). Only animals that satisfy both criteria are dispensed into wells for drug screening.

Figure 13.5.

Image acquisition using the ArrayScanV^{II}. Brightfield (*left*), mCherry (*center*), and GFP (*right*) images of transgenic worms expressing an aggregation-prone serpin-GFP fusion. The SpotDetector BioApplication is used to automatically quantify the number, area, and intensity of GFP-positive, serpin aggregates (green spots) within each worm. Images obtained (in part) from Gosai *et al.*, 2010.

Table 13.1

List of useful *C. elegans* resources

Source	Mission	URL for searching information	Comments
Wormbase	Major repository for <i>C. elegans</i> information including genomic, genetic, anatomy, and literature	http://www.wormbase.org	Very helpful resources including: <ul style="list-style-type: none"> • Gene expression patterns • Genetic map information • Mutant phenotypes • Complete Genome sequence • Developmental lineage of the worm
Caenorhabditis Genetics Center (CGC)	Collects, maintains, and distributes stocks of <i>C. elegans</i>	http://www.cbs.umn.edu/CGC/	<ul style="list-style-type: none"> • Thousands of strains available • Constantly being updated • Strains available to research community for nominal fee
Caenorhabditis elegans Gene Knockout Consortium (OMRF)	Production of deletion alleles of specified gene targets	http://celeganskoonsortium.omrf.org/	<ul style="list-style-type: none"> • Investigators can submit request for generation to knockout mutants • Mutants deposited into CGC
<i>C. elegans</i> National Bioresource Project of Japan (NBRP)	Collect nematode bioresources including: making and distributing deletion mutants	http://www.shigen.nig.ac.jp/c.elegans/index.jsp	<ul style="list-style-type: none"> • Free with MTA • Simple website to search for deletions • Over 4000 deletions available
NCBI GenBank, Blast	Repository of sequences from many diverse organisms with extensive blast services	http://www.ncbi.nlm.nih.gov/Genbank/index.html http://www.ncbi.nlm.nih.gov/BLAST/	<ul style="list-style-type: none"> • Wealth of information • Free access • Centralized collective archive • Blast easily used to find similarities between sequences
WormAtlas	Provides anatomical information	http://www.wormatlas.org	<ul style="list-style-type: none"> • Simple text search • Excellent handbook on worm anatomy
<i>C. elegans</i> Movies	Provides a visual introduction to a <i>C. elegans</i>	http://www.bio.unc.edu/faculty/goldstein/lab/movies.html	Good introductory videos about worm development, phenotypical studies, and techniques
Addgene	Archives and distributes plasmids	http://www.addgene.org	<ul style="list-style-type: none"> • Large variety of plasmids available

Source	Mission	URL for searching information	Comments
			<ul style="list-style-type: none">• Vector kit available contain 288 vectors designed for <i>C. elegans</i> research
BioScience Life Science (formerly GeneService)	Provides RNAi feeding library originally constructed by Julie Ahlinger's group	http://www.lifesciences.sourcebioscience.com/	<ul style="list-style-type: none">• Library covers 87% of <i>C. elegans</i> genes• Library can be purchased whole or in part
BioScience Life Science	<i>C. elegans</i> Fosmid Library	http://www.lifesciences.sourcebioscience.com/	<ul style="list-style-type: none">• Offers complete clone set or individual clones• <i>C. elegans</i> FOS finder tool that allows you to search for your gene or region of interest
<i>C. elegans</i> www server	<i>C. elegans</i> resource center	http://elegans.swmed.edu/	<ul style="list-style-type: none">• Offers links to important <i>C. elegans</i> resources such as literature searches, software, genome, etc.

Table 13.2

Common promoters used for transgenesis

Promoters	Expression location
<i>nhx-2, vha-6</i>	Intestinal cells
<i>myo-2</i>	Pharyngeal muscles
<i>myo-3</i>	Body muscles
<i>pes-10</i>	Embryo
<i>hsp-4, hsp-16</i>	Heat/stress-inducible expression
<i>pie-1</i>	Germline
<i>cdh-3</i>	Anchor cells
<i>ttx-4</i>	Sensory neurons
<i>unc-119</i>	Pan-neuronal
<i>unc-122</i>	Coelomocytes
<i>lin-26</i>	Epithelial tissues, gonad, and uterus

Table 13.3

Coinjection markers for DNA transformations in *C. elegans*

Coinjection marker	Description	Advantages	Disadvantages
<i>myo-2::FP</i>	Drives FP expression in pharynx	<ul style="list-style-type: none">• Dominant visible marker• Transgenic worms easily identified under a fluorescent dissecting scope• Useful for genetic screens and mating• Promoter expressed through all stages of development	<ul style="list-style-type: none">• Requires fluorescent dissecting scope• High concentration of plasmid can cause expression of fluorescent protein in coelomocytes
<i>myo-3::FP</i>	Drives FP expression in body wall muscles	<ul style="list-style-type: none">• Same as above	<ul style="list-style-type: none">• Requires fluorescent dissecting scope• Could interfere with expression studies of other fluorescent fusions
<i>rol-6</i>	Dominant roller	<ul style="list-style-type: none">• Transgenic worms will have a dominant <i>roller</i> phenotype• No need for a fluorescent dissecting scope	<ul style="list-style-type: none">• Suppressed in some mutant backgrounds (uncoordinated and dumpy)• Roller phenotype not visible before L2 stage
<i>unc-119</i> (rescue)	Used in conjunction with <i>unc-119(ed3)</i> worms	<ul style="list-style-type: none">• Nontransgenic worms are uncoordinated and do not move• Only transgenic animals will move freely	<ul style="list-style-type: none">• Requires the use of <i>unc-119</i> mutant strain
<i>pha-1</i> (rescue)	Used in conjunction with <i>pha-1(E2123)</i>	<ul style="list-style-type: none">• Nontransgenic worms embryonic lethal at 25 °C• Only transgenic worms will survive at 25 °C	<ul style="list-style-type: none">• Requires the use of <i>pha-1</i> mutant strain
<i>unc-22</i>	Dominant twitcher	<ul style="list-style-type: none">• Transgenic worms will display a dominant <i>twitching</i> phenotype	<ul style="list-style-type: none">• Phenotype can be subtle and sometimes difficult to detect