Chapter 5

Whole-Animal High-Throughput Screens: The *C. elegans* Model

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Summary

The nematode *Caenorhabditis elegans* shows a high degree of conservation of molecular pathways related to human disease, yet is only 1-mm long and can be considered as a microorganism. Because of the development of a simple but systematic RNA-interference (RNAi) methodology, *C. elegans* is the only metazoan in which the impact of "knocking-down" nearly every gene in the genome can be analyzed in a whole living animal. Both functional genomic studies and chemical screens can be carried out using *C. elegans* in vivo screens in a context that preserves intact cell-to-cell communication, neuroendocrine signaling, and every aspect of the animal's metabolism necessary to survive and reproduce in lab conditions. This feature enables studies that are impossible to undertake in cell-culture-based screens. Although genome-wide RNAi screens and limited small-molecule screens have been successfully performed in *C. elegans*, they are typically extremely labor-intensive. Furthermore, technical limitations have precluded quantitative measurements and time-resolved analyses.

In this chapter, we provide detailed protocols to carry out automated high-throughput whole-animal RNAi and chemical screens. We describe methods to perform screens in solid and liquid media, in 96 and 384-well format, respectively. We describe the use of automated handling, sorting, and microscopy of worms. Finally, we give information about worm-adapted image analysis tools to quantify phenotypes. The technology presented here facilitates large-scale *C. elegans* genetic and chemical screens and it is expected to help shed light on relevant biological areas.

Key words: 384-well plate, 96-well plate, Agar, Antimicrobial, Automation, *C. elegans*, Chemical, Fluorescent marker, High-throughput, In vivo, Quantitative, RNAi, Screen, Whole-animal.

1. Introduction

Caenorhabditis elegans provides an ideal compromise between complexity and tractability. Many biological processes are conserved between humans and *C. elegans* to such an extent that

P.A. Clemons et al. (eds.), *Cell-Based Assays for High-Throughput Screening, Methods in Molecular Biology, Vol. 486*

DOI: 10.1007/978-1-60327-545-3_1

C. elegans data in many cases predict gene function, drug–target interaction, and target validation in mammals *(1–3)*. *C. elegans* investigations have already fostered a better understanding of the underlying mechanisms of a number of human diseases *(4–6)*. Moreover, although *C. elegans* is a sophisticated multicellular animal, all 959 somatic cells of its transparent body are visible with a microscope. Its constant body pattern, in combination with fluorescent markers, makes almost any aspect of *C. elegans* physiology suitable for in vivo study. Furthermore, an emerging set of informatic imaging technologies, such as WormTracker, allows automated behavioral analysis *(7)*.

In 2003, *C. elegans* became the first multicellular organism for which systematic RNAi experiments could be performed in a living animal *(8)*. siRNA can be delivered systemically simply by feeding *C. elegans* an *E. coli* strain expressing a double-stranded RNA (dsRNA) targeting a gene of interest *(9)*. The availability of a genome-wide RNAi feeding library has facilitated the completion of multiple genome-wide loss-of-function phenotypic screens *(10–12)*. Furthermore, by comparing the effects of RNAi in wild-type and mutant backgrounds, *C. elegans* allows large-scale analysis of functional genetic interactions *(13)*. Finally, the conservation of disease pathways and the cost-effectiveness of its cultivation make *C. elegans* an incomparable tool to discover new bioactive compounds and their targets. The combination of traditional genetics, RNAi technology, and small-molecule screens can help triangulate on potential drug targets, a major issue with any drug screen *(14)*.

Despite the potential value of *C. elegans* for gene, drug, and drug-target discovery, current screens are carried out manually, are extremely labor-intensive, use volumes incompatible with high-throughput chemical screens, and are not quantitative.

Here we present two protocols for automated and quantitative high-throughput (HT) *C. elegans* screening. The described methods enable quantitative analyses of a wide range of biological processes, such as the response to different types of biotic (pathogens) or abiotic (heavy metals, ultraviolet radiation, heat) stresses that affect viability, as well as traditional longevity studies. In addition, automated examination of any phenotypic readout based on fluorescent markers such as green fluorescent protein (GFP), Nile Red, or MitoTracker becomes feasible. Finally, the study of chemical or genetic perturbations that affect growth rates or body size could also be automated using the described methodologies.

To improve the throughput of RNAi screens, we developed a protocol to miniaturize the standard *C. elegans* RNAi screening method that is typically carried out in 6 or 12-well plates. In the presented HT assay, worms are grown in 96-well plates containing solid media, which prevents the metabolic shift

manifested as slow growth and a starved aspect that occurs when *C. elegans* is grown or transferred to a liquid medium. Briefly, RNAi-feeding bacteria are grown overnight and then seeded in 96-well microtiter plates containing low-fluorescent agar media with IPTG to induce the synthesis of double-stranded RNA. In two-generation screens, two synchronized first larval stage (L1) worms, obtained by bleaching adults and allowing the embryos to hatch overnight without food, are dispensed into each well using a worm-handling robot. The plates are incubated for six days, allowing sufficient time for the two L1 worms to grow to adults, to lay eggs, and to allow the eggs to hatch and develop. In one-generation screens, twenty synchronized L1s are dispensed in each well and are incubated for three days to allow the 20 animals to reach adulthood. Images of wells are taken with an automated microscope and then analyzed with adapted image-analysis tools.

To enable chemical screens, we developed a method to screen for small molecules in 384-well plates. The described assay was designed to screen low-molecular weight compounds that prevent the lethal effect of infection by the bacterial pathogen *Enterococcus faecalis (15)*. *C. elegans* are first grown to the young adult stage and then infected on the lawns of *E. faecalis*. The infected worms are washed and transferred to 384-well plates containing liquid media and the compounds to be tested. The plates are incubated until the infection kills untreated worms. The worms are washed and stained with a fluorescent dye that specifically stains dead worms. Images of the wells are captured with an automated microscope and analyzed to quantify worm survival.

1.1. Initial General Considerations Regarding RNAi and Chemical Screens

There are a number of characteristics that make an assay suitable for HT approaches involving *C. elegans*. The assay must be robust, reproducible (high Z`-score; *see* **Chap. 1**), and have a readout that is amenable to automated analysis. In addition, it must be possible to miniaturize the assay to a 96-well or higher-density format. Many variables need to be optimized to develop an assay, including genetic background, readout, food source, temperature, timing, number of animals needed to have sufficient statistical power, number of replicates, *etc*. All those variables will need to be adjusted to the 96 or 384-well format, where small perturbations such as salt concentration, amount of food, and number of animals will have a larger effect than in a standard assay, given the limitations that the small format imposes. An important feature of HT *C. elegans* assays is that sterile working conditions need to be maintained at all times, given that growth and handling of worms is carried out in pools and therefore contamination introduced at one step can ruin numerous assay plates.

2. Materials

2.1. RNAi Screens

2.1.1. Reagents

1. *C. elegans* strain NL2099 (*rrf*-3 (*pk1*426)) *(16)*. Worms can be obtained from the *C. elegans* Genetics Center (http://www.cbs.umn.edu/CGC/Strains/request.htm) (*see* **Note 1**).
2. RNAi feeding library constructed in the Ahringer laboratory is available from Geneservice, Ltd. (http://www.geneservice.co.uk/products/rnai/index.jsp).
3. Potassium phosphate buffer, pH 6.0: 108.3 g KH_2PO_4, 35.6 g K_2HPO_4, water to 1 L. Sterilize by autoclaving.
4. NGM agar: 3 g NaCl, 2.5 g peptone, 17 g agar, 975 mL water. Autoclave. Cool to 55°C. Add in order the following sterile solutions: 1 mL of 5-mg/mL cholesterol dissolved in ethanol, 1 mL of 1 M $CaCl_2$, 1 mL of 1 M $MgSO_4$, and 25 mL of 1 M potassium phosphate, pH 6.0.
5. LB broth: 10 g Bacto-tryptone, 5 g yeast extract, 10 g NaCl, water to 1 L pH to 7.5 with NaOH. Sterilize by autoclaving. In some experiments LB is supplemented with 100 µg/mL of carbenicillin (LB-Cb).
6. LB-Amp/Tet plates: add 16 g/L of agar to LB-broth. Sterilize by autoclaving. After cooling, supplement with 100 µg/mL ampicillin and 25 µg/mL of tetracycline, and pour into Omnitray single-well square plates. Store at 4°C.
7. S-basal without cholesterol buffer (S-buffer): 5.85 g NaCl, 1 g K_2HPO_4, 6 g KH_2PO_4, water to 1 L. Sterilize by autoclaving.
8. *E. coli* OP50 bacteria: grow overnight in LB-broth, 200 rpm, at 37°C. Pellet bacteria, and concentrate 10 times in S-buffer (e.g., grow OP50 in 500 mL of LB-broth and resuspend in 50 mL of S-buffer). Store at 4°C. When needed seed 1 mL of OP50 suspension per 90 mm NGM agar plate and let dry.
9. Lysis solution: 195 µL 10N NaOH, 600 µL sodium hypochlorite (Aldrich; St. Louis, MO) or commercially available bleach brands, water to 3 mL.
10. Low-fluorescent RNAi media (LFR-media): 2 g NaCl, 2 g bactopeptone, 16 g agarose, water to 1 L. Sterilize by autoclaving with a magnetic bar per flask. After autoclaving, add in order the following sterile solutions: 1 mL of 5-mg/mL cholesterol dissolved in ethanol, 1 mL of 1 M $MgSO_4$, 25 mL of 1 M potassium phosphate, pH 6.0. Cool to 60°C and then add 1 mL of 1-*M* $CaCl_2$, 1 mL of 100-mg/µL carbenicillin, and 5 mL of 1 M IPTG (isopropyl-β-D-thiogalactopyranoside) (*see* **Note 2**).
11. Sytox® green (Invitrogen; Carlsbad, CA).

2.1.2. Supplies and Equipment

1. Omnitray single-well square plates (Nunc Nalgene International; Rochester, NY).
2. 96-pin replicator (Nunc Nalgene International).
3. Deep-well 96-well plates (Corning Costar; Lowell, MA).
4. Breathe-Easy sealing membranes (Sigma-Aldrich; St. Louis, MO).
5. 37°C shaking incubator with holders for 96-well plates (Fisher Scientific; Pittsburgh, PA). One holder allows stacking up to 3 deep-well 96-well plates.
6. Titertek MAP-C2 Agar Dispenser System (http://www.titertek.com).
7. Black clear-bottom half-area 96-well plates (Corning).
8. Benchtop centrifuge with 96-well plate adaptors (Beckman Coulter Allegra X-15R with Beckman Coulter SX4750 adaptors).
9. Thermo Multidrop Combi and CyBio CyBi Well Vario liquid dispensers or equivalent.
10. Vertical flow biological hood.
11 COPAS BIOSORT (Harvard Bioscience; Holliston, MA).
12 12–25°C ThermoForma worm incubators.
13 Molecular Devices Discovery-1, or equivalent automated microscope, with bright-field transmitted light option and appropriate fluorescent filter sets.
14 CellProfiler program; freely available at www.cellprofiler.org.

2.2. Chemical Screens

2.2.1. Reagents

1. LB-broth: *see* **Subheading 2.1**.
2. NGM agar: *see* **Subheading 2.1**.
3. SK-NS agar: 3 g NaCl, 3.5 g peptone, 20 g agar, 975 mL water. Autoclave. Cool to 55°C. Add in order the following sterile solutions: 1 mL of 5-mg/mL cholesterol dissolved in ethanol, 1 mL of 1 M $CaCl_2$, 1 mL of 1 M $MgSO_4$, and 25 mL 1 M potassium phosphate, pH 6.0, 1 mL of 100-mg/mL streptomycin sulfate, 1 mL 62.5 U/μL nystatin (*see* **Note 3**).
4. M9: 6 g Na_2HPO_4, 3 g KH_2PO_4, 5 g NaCl, 0.25 g $MgSO_4$–$7H_2O$, water to 1 L. Autoclave.
5. 5 M NaOH.
6. Bleach; commercially available brands are suitable.
7. *C. elegans strain glp-4(bn2);sek-1(km4) (17)*. Propagate at 15°C (*see* **Note 4**).
8. Brain-Heart Infusion (BHI) broth and agar media (BD; Sparks, MD): 52 and 37 g/L, respectively. Autoclave, cool and supplement with 100 μg/mL kanamycin sulfate.

9. *E. faecalis* strain MMH594 (cytolysin^{+}, gentamycin^{R}) *(18)*.
10. Bleach solution consisting of 0.1 mL of 5 M NaOH and 0.4 mL bleach.
11. 1.75X media: 35% BHI, 63.4% M9, 1.25% DMSO, 109 U/mL nystatin, and 175 μg/mL kanamycin sulfate.

2.2.2. Supplies and Equipment

1. Black-walled clear-bottom 384-well plates (Corning).
2. Gas-permeable membranes (Diversified Biotech; Boston, MA).
3. COPAS BIOSORT (Harvard Bioscience).
4. Sytox Orange (Invitrogen).
5. Bio-Tek ELx405 (Bio-Tek; Winooski, VT) or equivalent microtiter plate washer with an adjustable-height aspiration manifold.
6. Thermo Multidrop Combi (Thermo; Milford, MA) or equivalent liquid dispenser for 384-well microtiter plates.
7. Large orifice 1–200 μL pipet tips (Fisher Scientific).
8. Discovery-1 automated microscope (Molecular Devices; Sunnyvale, CA) or equivalent.
9. CellProfiler program; freely available at www.cellprofiler.org.

3. Method

3.1. RNAi Screens

3.1.1. Timeline and Throughput

The maximum number of plates manageable per experiment depends on personnel, equipment, worm strain, and particularities of the phenotype of interest. In our setting, image capture is the limiting step (Discovery-1 automated microscope; Molecular Devices). We can process up to thirty 96-well plates in 10 h (**Fig. 1**).

3.1.2. Amplify Worm Stocks

Incubation time and temperature, as well as brood size, vary with the strain. In the case of NL2099 worms, each 90-mm NGM-agar plate containing 5,000 gravid adults in their peak of egg-laying will produce enough eggs to seed 20 live hatchlings per well, in five 96-well plates (*see* **Note 1**).

3.1.3. Replicate RNAi Clones

Replicate the RNAi bacterial glycerol stocks onto agar LB-Amp/Tet single-well square plates using the following procedures.

1. Remove the tips and holders from three 200-μL tip boxes in order to use the boxes as containers.
2. Label and fill box 1 with 10% bleach, box 2 with sterile water, and box 3 with 96% ethanol.

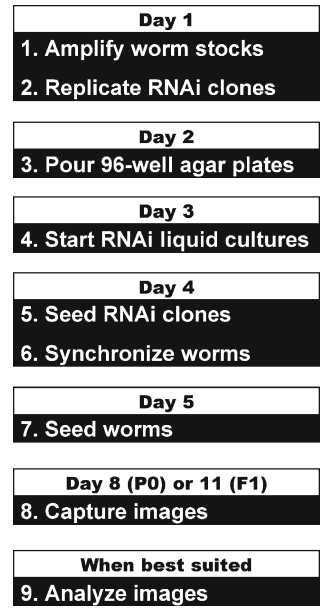

Fig. 1. RNAi screen timeline. Numeration used as in **Subheading 3.1**, where procedures are described in detail.

3. Soak the 96-pin replicator in bleach for 15 s, rinse in sterile water bath, dip in ethanol, and flame thoroughly.
4. Let the replicator cool down, firmly press onto the glycerol stocks (do not let them thaw), and then softly touch the agar plates.
5. Incubate overnight at 37°C.
6. Store agar plates with grown RNAi-feeding bacteria at 4°C (*see* **Note 5**).

3.1.4. Pour 96-Well Agar Plates

1. Prepare 20 mL of LFR media per 96-well plate, and an additional 100 mL to prime the agar dispenser. Keep agar at 60°C.
2. Autoclave 2 L of ddH_2O and keep warm at 85°C. Sterilize two 500-mL beakers with stir-bars in them.
3. Under sterile conditions, remove the lids and stack up to twenty-nine half-area 96-well plates (add a spare plate to the bottom and cover the top plate with a lid to avoid contamination). Maintain lids in sterile conditions. Use the restack function of the MAP-C2 agar dispenser to restack the plates; this function will minimize misalignment of the plates.
4. Sterilize the MAP-C2 agar dispenser tubing system by running 10 cycles with 96% ethanol. Wash out the ethanol and warm the system by running 5 cycles of hot ddH_2O. Transfer 250 mL at a time of LFR media to a sterile 500-mL beaker

(keep constantly stirring at 60°C). Prime the system with LFR media.

5. Pour 100 µL of media per well of a half-area 96-well plate.
6. Remove the plates from the stacker. This step can be done immediately, but be careful to avoid spilling the media. Put the lids back on the plates under sterile conditions. Let the media solidify for 1 h. Invert plates and leave overnight on the benchtop (*see* **Note 6**).

3.1.5. Start RNAi Cultures

Use a Thermo Multidrop Combi liquid handler to fill deep-well 96-well plates with 1.2 mL of LB-Cb. Use the same procedure as in **Subheading 3.1.3** to sterilize thoroughly the 96-pin replicator and manually replicate RNAi-feeding bacteria from agar LB-Amp/Tet square plates into deep-well 96-well plates containing 1.2 mL of LB-Cb. Seal plates with Breathe-Easy membranes (remove the second layer of the membrane). Stack plates in 96-well plate holders. Incubate for 8–14 h at 37°C, 200 rpm (longer times lead to reduced efficiency) (*see* **Note 7**).

3.1.6. Seed RNAi Clones

1. Label or barcode all plates.
2. Centrifuge the cultures grown in deep-well 96-well plates for 5 min at 2,000 × g.
3. Remove the supernatant (*see* **Note 8**).
4. Using the Thermo Multidrop Combi liquid handler with a standard cassette set to dispense into a deep-well 96-well plate, add 100 µL of S-buffer/well. Using a CyBio CyBi Well Vario with a 96-well head adaptor, resuspend bacteria using 5 cycles of pipetting at speed of 300 rpm. Seed 25 µL of bacteria in two independent plates (duplicates). Leave plates on benchtop until drying starts (*see* **Note 9**).
5. Dry plates in vertical-flow biological hood for 14 h (*see* **Note 10**).

3.1.7. Synchronize Worms

1. Harvest gravid adults and laid eggs of up to four 90-mm plates with 2.5 mL of S-buffer per plate, and transfer them to 15-mL conical tubes. Centrifuge at 1,500 × g for 30 s to pellet worms and eggs (all centrifugation steps are performed using the same conditions). Remove the supernatant by aspirating the liquid with a sterile glass Pasteur-pipette attached to a vacuum line (every time the supernatant is removed, it should be done this way). Resuspend the pellet in 3 mL of S-buffer.
2. Add 3 mL of freshly prepared lysis solution. Shake and vortex the tube for 1 min. Quickly fill tube with S-buffer and centrifuge. Remove 12 mL of supernatant. Add 3 mL of lysis solution to the 3 mL of lysate remaining, vortex and shake for 1–2 min. Quickly fill tube with S-buffer and centrifuge. Remove the

supernatant, leaving as little volume as possible without disturbing the pellet. Repeat the washing step (fill the tube, centrifuge, and remove all the supernatant) at least three times. Do all washing steps under sterile conditions (*see* **Note 11**).

2. Resuspend the eggs and embryos in 5 mL of S-buffer and let them hatch for no less than 18 h at RT with gentle rocking. Hatched worms will arrest at the first larval stage (L1) in the absence of food.

3.1.8. Seed Worms

Worm Preparation in order to separate worm and agar debris from L1 larva, centrifuge for 3 min at 150 × g. Transfer the supernatant containing the synchronized L1s to a fresh tube (leave the bottom 250 µL of liquid and discard the tubes). Pool suspensions and dilute or concentrate to have 10 L1s/µL. At least 1 h before seeding the L1s, add 1 µM Sytox® green (final concentration) (*see* **Note 12**). Leave worms gently rocking at RT until seeding. The combination of Sytox® green with the use of a worm sorter (COPAS Biosort) allows dispensing only live L1s in the 96-well plates.

1. COPAS Biosort Preparation: Sterilize the worm sorter tubing system by running 200 mL of 10% bleach. Wash out the bleach by running 200 mL of sterile ddH_2O. Run 200 mL of 70% ethanol. Wash out the ethanol by running 200 mL of sterile ddH_2O. Equilibrate with sample buffer, by running 50 mL of S-buffer.
2. Add 200 mL of S-buffer and 2 mL of Sytox® green-stained L1 suspension to the sample cup. Perform an acquisition cycle of 500 objects. For accurate dispensing of L1s, the flow rate should be approximately 10 events/sec. Dilute or add L1 suspension accordingly. Based on size and intensity of the fluorescent signal, define the gate of interest (**Fig. 2**). Perform a test by dispensing 5 non-green objects (live L1s) per well in the cover of a 96-well plate. Check accuracy under the dissecting scope (*see* **Note 13**).

3.1.8.1. Sorting for one-Generation Screens

Seed 20 live (non-green) L1s per well of a 96-well plate. Dry plates for 1 h in biological hood.

3.1.8.2. Sorting for Two-Generation Screens

Seed two live (non-green) L1s per well of a 96-well plate.

1. One-generation screens: Incubate for 3 days at 20°C, then shift to 25°C. Next day, worms on control RNAi will be adults laying unfertilized oocytes (thermosterile background).

3.1.9. Incubation

2. Two-generation screens: Incubate for 84 h at 20°C and then transfer to 25°C. At this time NL2099 worms on control RNAi will have laid approximately 10 eggs. After shifting to 25°C, NL2099 worms will lay approximately 20 more eggs before they start laying unfertilized oocytes. Maintain the

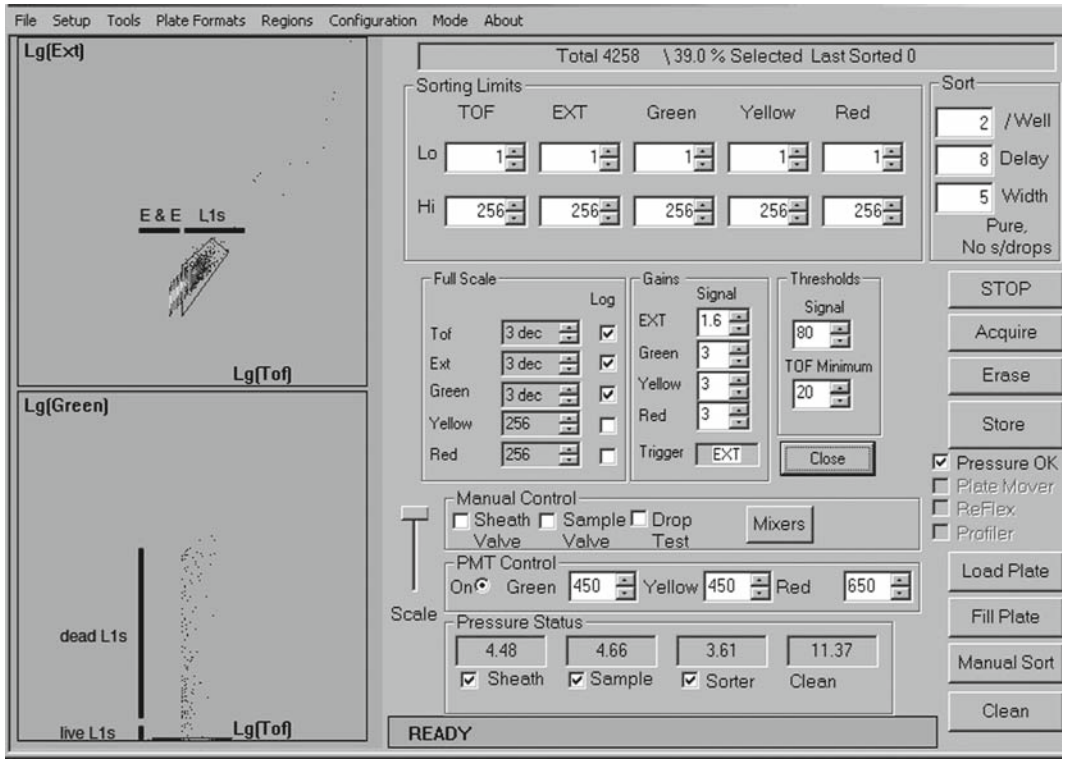

Fig. 2. Optimized COPAS Biosort conditions for sorting L1s. The post-run interface is depicted. The *upper dot-plot* shows the distribution of a post-egg prep population. The extinction or density (EXT) vs. the time of flight or length (TOF) is graphed. The *gated area* includes mostly L1s (dead and live) and excludes most of the non-hatched eggs and embryos (E & E). The *lower dot-plot* shows the distribution of the L1 subpopulation when the TOF vs. the green fluorescent intensity is graphed. The gated area includes live (*non-green*) L1s. In addition a tail of dead (Sytox® green positive) L1s is observed. Using the depicted settings, two live L1s, *non-green* gated objects, are sorted per well.

cultures at 25°C until the day on which scoring will occur. For most RNAi clones, the second generation will reach adulthood without starving (*see* **Note 14**).

3.1.10. Image Capture

Microscope: The major challenge to automate image capture in this format is imposed by the use of agar media. The background has high intensity and the focal plane changes from well to well because the media dries at different rates. An instrument able to perform image-based focusing is therefore indispensable. Capture settings will be different for different instruments and they will need to be adjusted for different 96-well plates and samples. The optimized parameters to capture bright-field, green-, and red-fluorescent signal with a Discovery-1 automated microscope (Molecular Devices) are described in **Table 1**. This setup allows imaging a half-area 96-well plate at three different wavelengths (bright-field, green-, and red-fluorescent signal) in 15 min (*see* **Note 15**).

Table 1
Image capture settings

Parameter	Setting
Plate Reference Point (a)	14337.5
Reference Objective (b)	5
Parfocality Offset (c)Offset	1,445
Plate Height (d)	14.2
Well Depth (e)	10,500
Find 2nd Maximum (f)	FALSE
Start z position (g)	19,982.5
Full range (μm) (h)	1,000
Full max step (μm) (i)	590
Plate bottom exposure (j)	3
Wide range (μm) (k)	50
Wide max step (μm) (l)	10
Accuracy (μm) (m)	59
Magnification	2×
Camera binning	2
Gain	2 (4×)
Transmitted light exposure	10 ms
Image based range (μm)	500
Max. step (μm)	100
Nile Red: filter set	572/630
exposure time	100 ms
GFP: filter set	470/530
exposure time	250 ms

Settings used to capture images of worms growing on half-area 96-well agar media plates with a Discovery-1 automated microscope are listed. Images are taken in 3 wavelengths: bright-field, GFP, and Nile Red.
(a) Reference point of flat sheet in plate holder. Value is distance from application z origin.
(b) Objective position used for setting reference point.
(c) Offset distance between current objective to reference-point objective.
(d) Height defined for current plate.
(e) Depth of well for current plate.
(f) TRUE = two-peak search, FALSE = single-peak search3
(g) Start z position of search in units.
(h) Total range covered in μm.
(I) Incremental steps in μm.
(j) Image exposure (ms).
(k) Search range at bottom of well in μm.
(l) Incremental steps in μm.
(m) Accuracy to which focus will be found (μm).

Samples on the scoring day, transfer plates to be documented to 12°C. This step will slow down growth, allowing the scoring of animals of the same age, and will help prevent starvation in two-generation screens. Take plates out of the incubator one at a time and capture images (*see* **Note 16**).

3.1.11. Image Analysis

Analysis is performed with CellProfiler (*see* **Chap. 15**). An optimized pipeline for green, red, and bright-field worm area analysis is available at www.cellprofiler.org.

3.1.12. Replication

RNA interference by feeding is highly variable; therefore, it is imperative to retest any putative phenotypes observed. We recommend retesting in 96-well plates and larger plate formats, so that a larger number of worms can be scored and phenotypes confirmed.

3.2. Chemical Screens

The protocol described below allows the screening of twenty 384-well plates per experiment. The rate-limiting steps are worm dispensing and imaging. Each of the limiting steps takes approximately 15 min per plate (**Fig. 3**).

3.2.1. Amplify Worm Stocks

1. Inoculate one colony of *E. coli* HB101 into a 2-L flask containing 500 mL LB supplemented with 200 μg/mL streptomycin sulfate. Incubate for 16 h at 37°C and 250 rpm. Centrifuge the saturated culture at 5,000 × g for 10 min and resuspend in

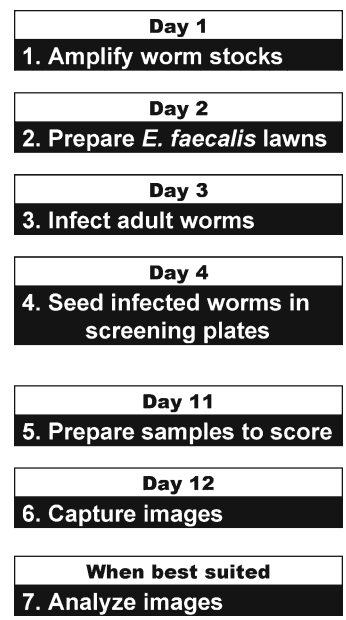

Fig. 3. Compound screen timeline. Numeration used as in **Subheading 3.2**, where procedures are described in detail.

LB to concentrate the bacteria 20-fold. Spread 100 µL of the concentrated bacteria onto 90-mm plates of NGM or SK-NS agar. Incubate the plates at RT for 1 day to produce the lawns of *E. coli* that will serve as the food source for the worms. Plates can be stored at RT for up to 1 week.

2. Grow approximately 1,000 *glp-4(bn2ts);sek-1(km4)* L1 worms on each 90-mm plate of HB101 on NGM at 15°C for 5 days until the worms become gravid adults.
3. Harvest the gravid adults by washing the plates with 10 mL of M9 and transfer them into 15-mL conical tubes. Centrifuge the tubes at 1,500 × g for 30 s to pellet the worms. Remove the supernatant, leaving behind 0.5 mL of liquid and the sedimented worms. Immediately before use, add 0.5 mL bleach solution to the tube. Continuously agitate and vortex the tube for 3–5 min, until most of the adult worms have ruptured. Do not overexpose to the bleach solution since this will result in damaged embryos. An average of ten to fifteen progeny per *glp-4(bn2ts);sek-1(km4)* adult will be obtained.
4. Wash the eggs 3 times with 14 mL M9 (*see* ***Subheading 3.1.7, steps 1–2** for wash procedures).
5. Resuspend the eggs in 5 mL M9 and let them hatch at RT for 20 h with gentle rocking. The hatched worms arrest at the L1 stage in the M9 buffer. Seed approximately 4,000 L1s onto each HB101 on SK-NS plate and grow the worms at 25°C for approximately 54 h until the worms become young adults.

3.2.2. Prepare E. faecalis Lawns

To prepare lawns of the pathogenic *E. faecalis* bacteria as a source of infection, inoculate one colony of *E. faecalis* strain MMH594 in BHI liquid media and incubate at 37°C for 6 h. Spread 100 µL of the culture over the entire surface of 90-mm plates containing BHI agar. Incubate the plates at 25°C overnight to grow the lawns and then cool the plates at 15°C.

3.2.3. Infect Adult Worms

To each plate of sterile, adult worms, add 15 mL of M9 and resuspend the worms by gently shaking the plate for 10 s. Transfer the worms into sterile 50-mL tubes. Allow the worms to settle to the bottom of the tubes and remove the supernatant. Wash worms twice with M9 to remove the *E. coli*.

1. Using large-orifice pipette tips, seed the worms onto the lawns of *E. faecalis* MMH594 on BHI agar. Transfer up to 8,000 animals per plate. Incubate the plates for 15 h at 15°C to allow the infection to become established (*see* **Note 17**).

3.2.4. Seed Infected Worms in Screening Plates

1. Using a multiplate dispenser, dispense 20 µL of 1.75X media to each well of the 384-well plate.

2. Pin transfer 100 nL of a 5-mg/mL compound stock solution (dissolved in DMSO) into each well of a 384-well plate (final DMSO concentration is 1% and compound concentration is 14 μg/mL).
3. Sterilize and prepare COPAS Biosort as described in **Subheading "COPAS Biosort preparation"** 3.1.8.1***.
4. Resuspend the infected worms in M9. Dispense 15 young adult worms into each well of the 384-well plates using the COPAS Biosort (*see* **Note 18**).
5. Dry the top of the 384-well plates with laboratory tissue to allow adhesion of membranes and seal the plates with gas-permeable membranes.
6. Incubate plates at 26.5°C with 85% relative humidity for 7 days (*see* **Note 19**). Place the plates in a single layer on top of the shelves and incubate without agitation.

3.2.5. Prepare Samples to Score

1. Resuspend the worms and bacteria by vortexing the plates for 5 s at a high setting. Centrifuge the plates at 1,000 × g for 10 s to remove the liquid from the membranes. Remove the membrane seals. Using a plate washer, dispense 65 μL of M9 per well using the maximum dispense speed to facilitate washing. Let the worms settle to the bottom of the wells for at least 3 min. Remove three-fourths of the liquid from the top of the plate using the aspirating head of the plate washer. Wash the plates a total of four times. After the final wash, aspirate enough liquid to leave ~ 25 μL/well (*see* **Note 20**).
2. Using the multiplate dispenser, dispense 50 μL of 1-μM Sytox Orange (diluted in M9) per well resulting in a stain concentration of 0.7 μM.
3. Seal the plates with gas-permeable membranes and incubate at 20°C for 20 h at 85% relative humidity. Arrange the plates in a single layer on the incubator shelves and incubate without agitation.

3.2.6. Image Capture

Wells are imaged using a Molecular Devices Discovery-1 automated microscope. Fluorescent TRITC (535 nm excitation, 610 nm emission) and transmitted light images are captured. Hardware capture conditions are as described in **Table 1**, except that the focal plane is set at the bottom of the well (laser based scanning). The use of a 2× objective allows capturing the entire area of a well in a 384-well plate.

3.2.7. Image Analysis

Analysis is performed with CellProfiler (*see* **Chap. 15**). An optimized pipeline to quantify worm survival (1 – (Sytox Orange worm area/bright-field worm area) = 1 – (dead worm area/total worm area)) is available at www.cellprofiler.org. An example of the quantification of worm survival using the described methodology is shown in **Fig. 4** (*see* **Note 21**).

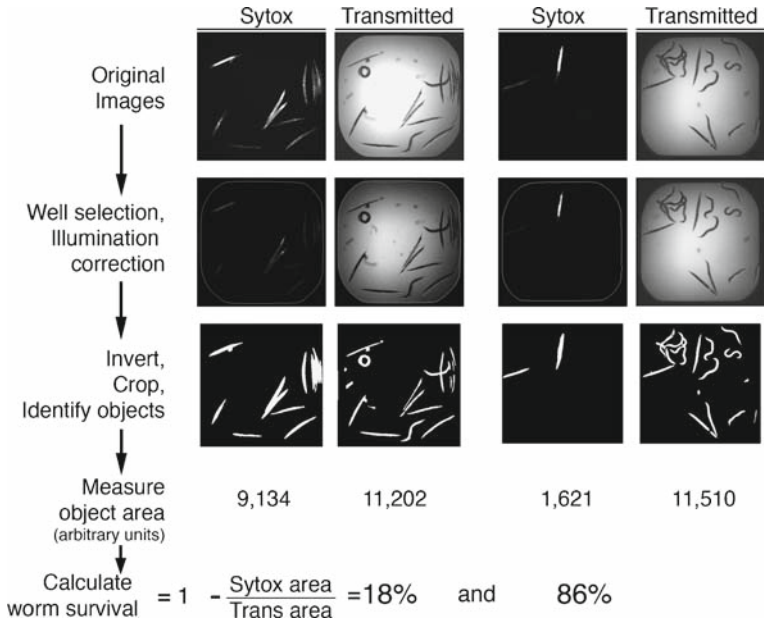

Fig. 4. Worm survival quantification using CellProfiler. Worms in 384-well plates are incubated with Sytox® orange, which specifically stains dead worms. *Top row* shows raw fluorescent Sytox® orange and bright-field images (captured using a ×2 objective) of an untreated well and an antibiotic-treated well. The images were analyzed using CellProfiler through a pipeline of 29 processing steps. The results of two of the processing steps are shown. Images in the *middle row* show the results of well-area selection and light correction of the bright-field images. Images in the *bottom row* show the result of the worm-identification function after inverting and cropping the well area. Finally, the total object area of the fluorescent and the bright-field images are measured. The areas of the Sytox® orange objects and bright-field objects are used to approximate the number of dead worms and the total number of worms, respectively. Worm survival = 1 – (Sytox® orange area/total bright-field worm area).

4. Notes

1. Finding the optimal worm genetic background is one of the most important aspects of developing a robust assay that can then be automated. We chose NL2099 because it carries a mutation in an RNA-dependent RNA polymerase (RRF-3), which makes this strain hypersensitive to RNAi *(16)*. The RRF-3 deficiency promotes stronger and additional loss-of-function phenotypes. In addition, *rrf-3* makes animals sterile at temperatures ≥ 25°C. The thermosterility allows the control of the number of progeny by shifting animals to 25°C. NL2099 does not have a significantly smaller brood size at 20°C when compared to wild-type animals, yet (as explained in **Subheading 3.1.8**) accurate dispensing of worms requires a large excess of synchronized progeny.

2. Carbenicillin and IPTG should not be stored for more than 2 weeks at 4°C. If starvation becomes a problem, adding 2 g of lactose/L allows for thicker lawns (S. Fischer, personal communication), but we have found that RNAi becomes less efficient for some phenotypes under these conditions. If interested in fat accumulation phenotypes, add 1/200,000 of 0.5-mg/mL Nile Red (in acetone).
3. Nystatin and streptomycin are added to inhibit fungal and bacterial contamination, respectively.
4. The *glp-4(bn2)^{ts} (19)* mutation makes the worms sterile when they are grown at the restrictive temperature of 25°C. Sterility is important in the infection assay because progeny production hinders the quantification of the initial population of worms. The sterile phenotype also prevents the matricidal hatching of progeny inside the parent worm that normally occurs in the absence of food. The *sek-1(km4)* mutant worms are more sensitive to pathogens *(17)* and its use shortens the incubation time of the assay.
5. Cross-contaminating RNAi-feeding bacteria is one of the most serious problems to which mishandling of stocks can lead. Extreme caution should be taken to thoroughly sterilize the replicator between plates. To maximize RNAi efficiency: Do not store replicated clones for more than four weeks; Do not replicate from agar plate to agar plate, or from liquid cultures to agar plates.
6. Alternatively, agar media can be manually dispensed. Keep media stirring at 60°C. Remove from two benchtop thermoblocks the metal racks normally used to hold tubes, thoroughly clean them with ethanol, and set them at 85°C. Place the 96-well plate to be filled with media in one thermoblock and the reservoir containing the media in the second block. Using a 12-channel pipettor, dispense 100 µL of media per well. Preheat the tips by slowly pipetting up and down 100 µL of media (two times). Dispense 100 µL of media slowly and carefully. Do not eject the last remaining bit of media to minimize bubble formation. Change tips with every plate or when bubbles appear.
7. Alternatively, use a 12-channel electronic pipettor and 1.2-mL sterile tips to fill deep-well 96-well plates with 1.2 mL of LB-Cb. Do not use less than 1.2 mL because the pin replicator will not touch the liquid if less volume is used. Alternatively, dispense 1 mL of LB-Cb and inoculate by using a multichannel pipettor.
8. In order to minimize contamination and/or cross-contamination of the samples, thoroughly clean, with bleach and ethanol, the sink area, and place three layers of clean paper towels next

to the sink. After centrifugation, quickly invert the plates to dispose the supernatant in the sink containing bleach, place the plates inverted on the paper towels to absorb the excess of liquid, and leave for 5 min.

9. Alternatively, using a 12-channel pipettor, manually add 100 μL of S-buffer to all wells of the deep-well plates containing the bacterial pellets. Resuspend bacteria and add 25 μL of RNAi bacteria per well in two agar 96-well plates (duplicate RNAi clones).
10. Drying conditions may vary from hood to hood. We found that adding 10 μL extra of water to columns 1 and 12, and to rows A and H, helps prevent the cracking of the media. We do not recommend drying in a horizontal air-flow hood because drying will be very uneven, leading to cracked outer wells and wet inner wells.
11. Different labs use different synchronization protocols. We present two versions in this chapter. **Subheading 3.1.7** describes a double-bleaching protocol that helps to dissolve most of the carcasses that could later interfere with dispensing or imaging of the worms. In addition, **Subheading 3.1.7** uses S-buffer instead of M9. The S-buffer salt content is much lower than that of M9. If M9 is used in half-area 96-well plate assays, the final concentration of salts after drying the plates is too high and appears to affect worm growth rates.
12. Sytox® green is a membrane-impermeable dye that stains nucleic acids in dead and dying cells. In an L1 population synchronized by bleaching, it will stain dead eggs, embryos, and L1s.
13. In our hands, 5% of the wells will have ±1 worm. When dispensing fewer than five L1s per well, use the "pure" mode. The use of the pure mode allows maximum accuracy, but it requires a large excess of animals because most of the objects will not fulfill the sorting specifications (*see (20)* for more details on sorting conditions and requirements). When dispensing five or more L1s per well use the "enhanced" mode.
14. A caveat of this approach is that the second generation of the slow-growing RNAi clones will not be scorable because plates will be shifted to 25°C before they would have started laying eggs.
15. Useful general tips: Use a different starting z-position for external (rows A and H or columns 1 and 12) and internal wells; this will greatly reduce scanning time. Focus using bright-field imaging; this step will minimize the fleeing

behavior normally triggered in worms by short-wavelength light. Do not apply background subtraction or shadow correction; modifying raw images will have a negative impact on subsequent analysis.

16. If signal from a transgene is the readout of your assay, be aware that in some cases low temperatures can lead to transgene silencing.
17. Worms are infected on the lawns of pathogen for a period of time that allows persistent intestinal colonization, but at which symptoms are not yet observed.
18. The worm synchronization method described in **Subheading 3.2.1**, steps 3–4, results in a population of worms of which 95% are young adults. Based on TOF, the COPAS Biosort differentiates young adults from younger animals (**Fig. 2**). Fifteen young adults are dispensed per well of a 384-well plate. The remaining 5% of the worm population corresponds to slower-growing animals that are discarded. The COPAS Biosort transfers each worm in a volume of ~1 μL. The final volume per well is 35 μL. Final concentrations are as follows: 20% BHI, 36% M9, 1% DMSO, 100 μg/mL kanamycin sulfate, 62.5 U/mL nystatin, and the remaining liquid consists of sheath fluid (worm sorter specific fluid *(20)*) and M9.
19. Roughly 90% of the untreated worms die from the infection during the 7-day incubation period. In contrast, less than 15% of the worms die when treated with antibiotics such as ampicillin or tetracycline. Humidity is set at the maximum of the incubator capacity to reduce evaporation. Alternatively, the microtiter plates could be placed into containers that are lined with wet paper towels.
20. In addition to staining dead worms, Sytox® Orange also stains the bacteria in the assay wells and therefore the bacteria need to be removed to allow quantification of the fraction of dead worms.
21. We quantify survival by measuring areas (number of pixels) instead of counting worms because CellProfiler cannot distinguish overlapping worms as independent objects. The decision to measure area occupied by dead worms, instead of fluorescent Sytox Orange intensity was based on the observation that the intensity of the fluorescent-staining dead worms varied greatly. This variation, in addition to the inhomogeneous nature of the worm suspensions, also prevented the use of a fluorescent plate reader.

Acknowledgments

The authors thank Fred Ausubel, Harrison Gabel, and Jonah Larkins-Ford for critical review of the manuscript. We also thank Nicola Tolliday, Bridget Wagner, Lynn Verplank, Jason Burbank, and Anne Carpenter for intellectual and practical contributions to the development of the assays. This work was supported by a grant from the SPARC Program (Broad Institute of Harvard and MIT) to Gary Ruvkun, Fred Ausubel, Eyleen O'Rourke, David Sabatini, and Jonathan Clardy, and by NIH grant AI072508, Fred Ausubel PI.

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