Chapter 32

High-Throughput and Longitudinal Analysis of Aging and Senescent Decline in *Caenorhabditis elegans*

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Abstract

Caenorhabditis elegans is becoming a multipurpose tool for genetic and chemical compound screening approaches aiming to identify and target the molecular mechanisms underlying senescent decline, aging, and associated pathologies. In this chapter, we describe specialized methods that have been developed to facilitate such screening strategies using *C. elegans*. The first section provides detailed procedures for the assessment of lifespan parameters on liquid growth media that are typically used for rearing nematodes. In the second section, we consider methodologies optimized for high-throughput survival analysis, applicable to large-scale chemical compound or genetic screening ventures. Finally, we discuss recently developed microfluidics tools for the noninvasive monitoring of behavioral and physiological traits in longitudinal studies of aging and senescent decline.

Key words: Aging, *Caenorhabditis elegans*, Drug screening, Lifespan, Longitudinal studies, Microfluidics, Nematode, Senescent decline, Stochastic death, Survival analysis

1. Introduction

Caenorhabditis elegans is a model organism that is particularly suited for large-scale, high-throughput approaches to dissect and target mechanisms underlying senescent decline and age-associated pathologies. The small size and transparent body, coupled with a short lifespan and prolific clonal reproduction provide a unique combination of advantages that decisively facilitate such studies. In this chapter, we describe methods based on liquid worm cultures that build on these features and advantages of *C. elegans*, to monitor and quantify parameters and phenotypes associated with senescent decline and aging.

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The first method described here has proven particularly useful for nematode-based, high-throughput drug screening approaches (1–3). It allows lifespan assessment of *C. elegans* animals in 96-well plate liquid cultures. Survival assays in liquid media reproduce results obtained with lifespan experiments on solid media both for wild-type (N2) worms in different temperatures and for mutants strains with altered lifespan (3). Because this method of lifespan determination is relatively not labor-intensive and also amenable to automation, it can be readily integrated into automated drug screening platforms.

The increasing need for screening large libraries of chemical compounds to identify molecules that promote survival under different conditions (acute stress (4), bacterial infections (5)) led to the development of high-throughput methods for lifespan assessment. In this chapter, we describe a dedicated method or high-throughput analysis and screening of several thousands of compounds simultaneously, based again on liquid cultures in multi-well plates. This method uses survival after stress (a few hours) as a predictor of long-term lifespan (a few weeks) (4). Survival after stress is often linked to the normal lifespan of the animal. The connection between lifespan and stress resistance has been shown for different kinds of stressors (6–8). This approach is much shorter compared to performing lifelong survival analysis and can be easily combined with identification of drugs that affect worm survival after acute stress (thermal stress, oxidative stress, etc.). It can also be implemented for the examination of multiple genetic mutants simultaneously, to investigate how molecular pathways are implicated in stress responses (for an extended discussion of high-throughput screens in *C. elegans* see ref. 9).

The third method described in this chapter exploits a microfluidic device suitable for longitudinal studies and monitoring of age-related changes and senescent decline, in a noninvasive manner, throughout the lifetime of single animals. Detailed study of age-related changes is important because they reveal mechanisms implicated in the aging process, they indicate cause-and-effect relationships between these mechanisms and aging, and some may also be used as biomarkers for the assessment of the biological age of an organism. Microfluidics technologies and devices have recently been developed that greatly facilitate such analyses by allowing long-term observation of age-related traits in single animals (10). These devices can also be utilized in longitudinal studies that combine exposing of worms to chemical or other stressors at specific time points through life. They are also excellent tools for downstream analysis of chemicals or metabolites generated by the worm itself (for example pheromones).

2. Materials

2.1. Lifespan Assay in Liquid Media

- Luria Bertani (LB) medium and agar plates containing streptomycin: For 1 L, add 10 g Bacto-tryptone, 5 g yeast extract, 10 g NaCl. Adjust pH to 7.0 with NaOH, adjust volume to 1 L with dd H_2O, and autoclave. For LB agar plates add 20 g of agar to the previous mixture and a magnetic stirrer prior to autoclaving. After autoclaving let the mixture cool down to 55°C (with stirring when it is LB agar) add 1 mL of streptomycin stock 100 mg/mL and poor into petri dishes (10 mm diameter).
- 2 Streptomycin stock 100 mg/mL: For 10 mL, add 1 g of streptomycin in 10 mL of dd H_2O. Shake until the streptomycin is dissolved. Filer-sterilize the solution and store at 4°C.
- Nystatin suspension 10 mg/mL: For 50 mL, add 0.5 g nystatin, in 70% ethanol in dd H_2O. This is going to be a suspension, so it needs shaking prior to use (*see* **Note 1**).
- Hypochloride solution: For 10 mL, add 1 mL NaOH 5 N, 2 mL bleach, 7 mL double-distilled (dd) H_2O.
- M9 buffer: For 1 L, add 6 g Na_2HPO_4, 3 g KH_2PO_4, 5 g NaOH, 0.25 g $MgSO_4{\cdot}7H_2O$ (or 1 mL of $MgSO_4$ 1 M solution). Add dd H_2O up to 1 L and autoclave.
- Potassium Phosphate Buffer pH 6.0: For 1 L, add 136 g KH_2PO_4, adjust pH to 6.0 with 5 M KOH, add dd H_2O water up to 1 L and autoclave.
- Trace metal solution: For 1 L, add: 1.86 g Na_2EDTA, 0.69 g $FeSO_4{\cdot}7H_2O$, 0.20 g $MnCl_2{\cdot}4H_2O$, 0.29 g $ZnSO_4{\cdot}7H_2O$, 0.016 g $CuSO_4$. Add dd H_2O up to 1 L, autoclave, and store in the dark.
- Potassium citrate Buffer 1 M: For 1 L, add 268.8 g tripotassium citrate, 26.3 g citric acid monohydrate and dd H_2O up to 900 mL. Adjust pH to 6.0 with 5 M KOH, add dd H_2O up to 1 L and autoclave.
- S-basal medium: For 1 L, add: 5.9 g NaCl, 50 mL of 1 M Potassium Phosphate Buffer pH 6.0 (*see* above for details). Adjust the volume with dd H_2O up to 1 L and autoclave. Let the medium cool down and add 1 mL of 5 mg/mL cholesterol (dissolved in ethanol).
- S-complete medium: for 1 L, add: 977 mL S-basal medium, 10 mL 1 M Potassium citrate pH 6 (sterile) (*see* above for details), 10 mL Trace metal solution (sterile), 3 mL 1 M $CaCl_2$ (sterile), 3 mL 1 M $MgSO_4$ (sterile). Use sterile technique, do not autoclave.

- 0.6 mM Fluorodeoxyuridine (FUDR): For 67 mL, dissolve 10 mg FUDR in 67 mL sterile S-complete medium. Filter-sterilize the solution, aliquot and store at −20°C.
- Flat bottom 96-well plates.
- Plate mixer.
- Transparent adhesive plate sealers.
- *Escherichia coli* strain OP50-1.
- Incubator for stable temperature.

2.2. Automated High-Throughput Assessment of C. elegans Survival After Acute Stress

- COPAS Biosort instrument (Union Biometrica Inc., Holliston, USA).
- SYTOX Green fluorescent dye (Life Technologies Corporation, Eugene, Oregon, USA).
- Microtiter plate-reading fluorometer (Thermo Labsystems, Beverly, USA).
- Epi-fluorescence microscope.
- Black wall clear bottom 384-well plates.
- Transparent adhesive plate sealers.

2.3. Lifespan Assessment on a Chip: The Microfluidics Approach

- Microfluidic device: Photolithography is used to pattern raised features of SU-8 photoresist (Microchem Corp., Newton, MA, USA) on silicon wafer (Silicon Sense, Inc., Nashua, NH, USA). This silicon master serves as a template for replica molding with poly(dimethyl siloxane) (PDMS, Dow Corning Sylgard 184, Corning, NY). Subsequently, masters are treated with tridecafluoro (1,1,2,2 tetrahydrooctyl) trichlorosilane (Gelest, Inc., Philadelphia, PA, USA) (10).
- Polyethylene tubing.
- Syringe.

3. Methods

3.1. Lifespan Assay in Liquid Media

3.1.1. Preparation of Feeding Bacteria

1. An outline of this protocol is presented in Fig. 1. Starting from a freshly streaked culture of OP50-1 *E. coli* bacteria on an LB, streptomycin-containing agar plate, pick a single colony and inoculate 5 mL LB/streptomycin medium (see Note 2).
2. Incubate overnight (12–16 h) at 37°C, shacking at 250 rpm.
3. The following day, inoculate 500 mL LB/streptomycin using 500 μL of the overnight-saturated culture (1/1,000 dilution) and incubate at 37°C shacking for ~12 h until saturation.
4. Transfer the bacterial culture in a pre-weighted sterile centrifuge tube, and centrifuge for 10 min at 3,000 × *g*.

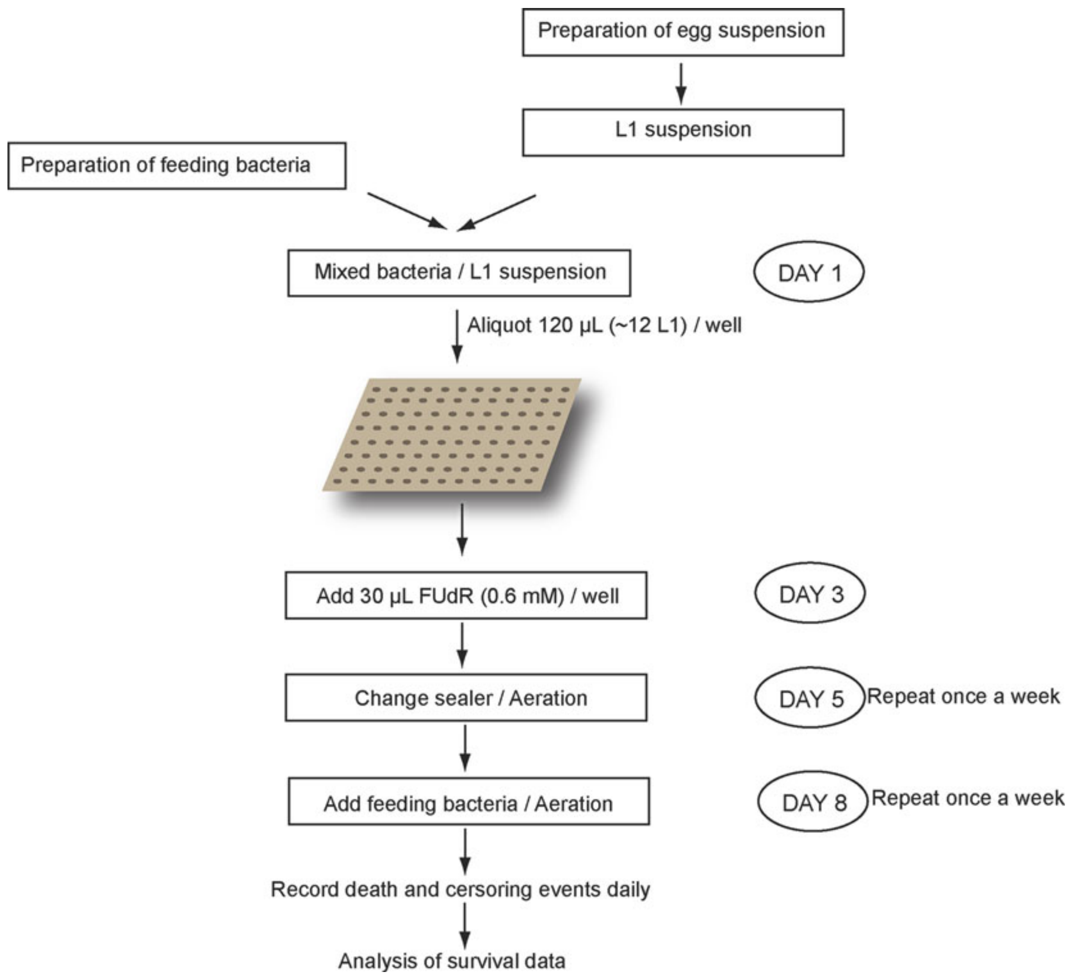

Fig. 1. Lifespan assay in 96-well plates. Briefly, this protocol involves the preparation of synchronous L1 larvae in S-complete medium, mixing them with bacterial suspension and dispersing them in the 96-well plate, at a concentration of ~12 worms per well. Just before larvae reach adulthood, FUdR is added to sterilize the population. Feeding bacteria are added once per week and the sealer is also changed once per week to allow aeration. Death and censoring events are recorded daily and analysis of the survival data follows the completion of the experiment.

5. Discard the LB medium and wash the cells twice with sterile water.
6. After the second wash, discard carefully all the remaining water and weight the cell pellet.
7. Resuspend the cell pellet in S-complete medium to a final concentration of 100 mg/mL (see Note 3). No cell clumps should remain after resuspension in S-complete medium. After adding the S-complete medium, resuspend the pellet by shacking for 15–60 min at 250 rpm at room temperature.
8. Resuspended bacteria can be stored at 4°C, for up to 2 weeks.

3.1.2. Population Synchronization

Preparation of synchronous worm populations for liquid cultures is easily accomplished by means of the egg-preparation technique (see Chapter 31).

1. Prepare 3 NGM agar plates with mixed worm population (see Note 4 and Chapter 31).
2. Collect worms by washing plates with 5–10 mL of sterile water (see Note 5) and transfer animals to a sterile tube.
3. Let worms settle to the bottom of the tube for about 5 min.
4. Remove supernatant and add 3 mL of freshly prepared sodium hypochlorite solution.
5. Vortex for about 1 min until worm bodies are dissolved and only eggs are obvious under the dissecting microscope.
6. Spin down the egg preparation for 2 min at $300 \times g$.
7. Wash the egg pellet three times with 5 mL of sterile water (see Note 6).
8. Wash eggs once with 5 mL of S-complete medium (see Note 6).
9. After the final centrifugation, resuspend eggs in 5 mL S-complete medium and transfer to a 50 mL sterile canonical tube.
10. Add another 35 mL of sterile S-complete medium and incubate overnight with gentle shacking on a nutator mixer.
11. Eggs will hatch during their overnight incubation in the S-complete medium. By next morning the tube will contain a suspension of L1 larvae (see Note 7).

3.1.3. Setting Up the Experiment

Preparation of L1 Larvae and Feeding Bacteria Suspension

1. Mix the L1 suspension by inverting the tube several times.
2. Spot 10 μL drops on NGM agar plates (make sure to mix directly before each pipetting). Measure the number of worms in each drop. Measure at least ten drops and estimate the mean number of worms per milliliter of suspension.
3. Adjust the final volume so that worm concentration in the suspension is approximately 100 worms/mL. If the estimated worm concentration is less than 100 worms/mL, spin down worms shortly and remove the appropriate amount of medium, to achieve the desired concentration.
4. Add streptomycin (to a final 200 μg/mL) and the antifungal drug nystatin (final concentration 10 μg/mL) to avoid contamination (see Note 2).
5. Add an appropriate volume of feeding bacteria in S-complete media (prepared in Subheading 3.1.1) to a final concentration of 6 mg/mL. The bacterial suspension will be further diluted by later adding the FUdR solution.

Transfer Animals to 96-Well Plates

It is important to use 96-well plate with transparent flat bottom.

1. Mix the worm suspension thoroughly by inverting the tube several times.
2. Disperse 120 μL of the suspension in each well. Mix the suspension often, because the larvae are quickly swimming downwards and their concentration on the surface changes.
3. Each well should contain approximately 12 L1 larvae.
4. Count worms in all wells and note the number of worms in each well.
5. Remove the wells that contain more than 18 worms from the assay. These wells are likely to run out of food earlier than the rest of the wells.
6. Seal the plate with a transparent adhesive sealer, to avoid contamination and evaporation of the samples.
7. Mix the plate on a micro-plate mixer for 2 min.
8. Put the plate in a 20°C incubator for 2 days.

Sterilize the Worms with FUdR

FUdR sterilizes L4 worms without significantly affecting adult physiology or aging (see Chapter 31). It can be used in a range of concentrations from 25 to 400 μM (11, 12). It should not be added to the culture before animals reach the L4 stage of development (approximately 2 days after the L1 larvae have been transferred to the 96-well plate), because it will affect their development (see Note 8).

1. Prepare an FUdR stock solution in S-complete medium at a concentration 0.6 mM.
2. Add 30 μL of the stock in each well containing late L4 larvae.
3. Seal again the micro-plate with an adhesive sealer.
4. Shake the plate for 2 min on a micro-plate shaker.
5. Return the plate back into the 20°C incubator.

Worms stop producing eggs within a few hours of FUdR addition. Eggs that will be already produced within this time frame will not develop normally in the presence of FUdR so they will not interfere with the assay by mixing with parents.

3.1.4. Feeding and Aeration of Worms in Liquid Cultures

To prevent starvation of worms, feeding bacteria need to be added once a week in each well.

1. Mix the feeding bacteria suspension kept in the refrigerator by inverting the tube several times.
2. Transfer an adequate fraction of the suspension into a fresh sterile tube and leave on the bench for 15–20 min to warm up to room temperature.

3. Remove the sealer from the micro-plate (next to a Bunsen burner to maintain sterile conditions).
4. Add 5 μL of bacterial suspension in each well and seal the plate again.
5. Mix the cultures for 2–3 min on a micro-plate shaker.
6. Return it into the 20°C incubator.

To allow fresh oxygen to enter culture wells, the tape sealer needs to be removed twice a week.

1. Remove the old sealer.
2. Leave the plate uncovered for 2 min, next to a Bunsen burner (to maintain sterile conditions).
3. Put a new sealer and shake for 2 min on a micro-plate shaker.
4. Return the plate into the 20°C incubator (see Note 9).

3.1.5. Scoring of Lifespan

Prepare a worksheet where the number of live, dead, and censored animals will be noted and monitor the cultures every 1–2 days.

1. Remove the plate from the 20°C incubator.
2. Mix the plate for 2–3 min to induce animal movement.
3. Monitor the plate every 1–2 days under the dissecting microscope without removing the tape sealer.

Scoring of live worms is based upon their motility and their body shape (5) (see Note 10). Live worms move (subtle movements can be visualized at higher magnification) and their body typically assumes a sinusoidal posture. Dead worms don't move and their body shape is usually like a rigid rod. Score and record the live and dead worms in each well every day. Do not remove dead animals from the assay wells. Some worms may appear as dead 1 day but then the next day they may move again.

3.1.6. Survival Data Analysis

The Kaplan–Meier analysis is the most common method used for processing and plotting survival data. The graph generated depicts the percentage of animals surviving at each observation point plotted against time. Several software applications perform survival data analysis based on the Kaplan–Meier method. Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA) is an extensively used application for a wide range of statistical analyses. Specific Excel add-ins can be obtained and installed for easier analysis of survival data (13). Origin is another software package (OriginLab Corporation, Northampton, USA) for scientific graphs and data analysis that is capable of generating Kalpan–Meier plots. GraphPad Prism (GraphPad Software Inc., San Diego, USA) is an additional, useful software application for scientific graphing that also includes survival curve construction and analysis. For further information on survival data analysis see Chapter 31.

3.2. Automated High-Throughput Assessment of C. elegans Survival After Acute Stress

In summary, synchronous worm populations of the same larval stage are selected by an automated large particle flow-sorting system and subsequently dispensed into 384-well plates containing a fluorescent dye, SYTOX green. Following stress, the dye stains only dead animals, and the overall survival of a population will be calculated based on the individual fluorescent curves that determine the time of death for each, single animal-containing well. The *fer-15(b26)* temperature sensitive sterile mutant strain can be used to avoid progeny interference (see Note 11).

3.2.1. Preparation of fer-15(b26) Sterile Populations

Solid Media

1. Grow mixed populations of *fer-15(b26)* mutant worms at 15°C to maintain fertility (see Note 4).
2. Transfer (with a wormpick) five young adult worms per plate, on 5 NGM agar plates (25 worms, total) and incubate at 25°C for 3–4 days. The progeny of *fer-15(b26)* worms will develop into sterile adults. The total number of worms needed depends on the experimental design. However, one NGM plate will hold approximately 1,500 worms, and ideally, the percentage of adult worms in the overall population should not exceed 25% at the beginning of the experiment.

If larger worm populations are needed, then preparation of a liquid culture is a more convenient alternative.

Liquid Media

1. Prepare feeding bacteria as described in Subheading 3.1.1, and three medium NGM agar plates with mixed worm population grown at 15°C to maintain fertility.
2. Add 475 mL of S-complete medium and 25 mL of feeding bacteria in a 2 L flask
3. Collect the worms from the NGM agar plates and prepare eggs by treatment with hypochlorite solution.
4. Add the eggs in the flask with the S-complete medium and the feeding bacteria.
5. Incubate at 25°C shaking at 160 rpm.
6. Worms should clear the bacteria from the culture in approximately 3–4 days (see Note 12).
7. Collect worms by tilting the flask and aspirating the supernatant after allowing animals to settle at the bottom.
8. Transfer worms in a sterile canonical tube.

3.2.2. Preparation of 384-Well Plate Cultures

Using 384-well plates not only multiplies the number of samples that can be assayed simultaneously but also improves the detection of signal coming from dead worms, as in 96-well plates worms can stay in the periphery of the well, introducing high variation in the detected results.

1. Inoculate 5 mL of LB/streptomycin with a single OP50-1 colony and incubate at 37°C shaking overnight.
2. In the next morning, add 50 μL of the saturated culture into 50 mL of fresh LB/streptomycin medium and incubate at 37°C, shaking until OD_{600} is about 0.2.
3. Spin down cells for 10 min at 3,000 × *g*.
4. Resuspend in equal volume (50 mL) of S-complete medium.
5. Wash twice in S-complete medium.
6. Add the antibiotics, streptomycin (200 μg/mL final concentration) and nystatin (10 μg/mL final concentration).
7. Dispense 20 μL of the bacterial suspension in S-complete medium in each well (see Notes 13 and 14).
8. Add SYTOX Green dye in each well at a final concentration 1 μM (see Note 13).

3.2.3. Automated Selection and Distribution of Age-Matched Individuals in 384-Well Plates with the COPAS Biosort Platform

Animals of different larval stages within a mixed population can be separated and sorted by large particle flow cytometry. The COPAS (Complex Object Parametric Analyser and Sorter; Union Biometrica Inc., Holliston, USA) Biosort platform has a specially engineered fluidic path and flow cell optimized to separate eggs, all larval stages and adults from mixed *C. elegans* populations. This system is capable of sorting worms using physical and optical properties, such as length and internal complexity (5, 14).

1. Once mixed population of sterile *fer-15(b26)* worms have been established, animals are collected in conical tubes and resuspended in M9 buffer containing 0.01% Triton X-100.
2. Wash 2–3 times in the same buffer.
3. Samples are passed through a 180 μM nylon mesh filter to remove larger clumps of eggs and debris.
4. Estimate the worm concentration in the sample (as described in Subheading 3.1.3). Adjust to 1 worm/μL using M9 (see Note 12).
5. Place the worm suspension to the sample cup a COPAS Biosorter, following the manufacturer's instructions, and proceed to dispense them. You may select both the developmental stage of worms in the assay and the number of worms per well.
6. Seal the plate with a transparent adhesive plate sealer to avoid evaporation and contamination.
7. Shake the plate using a plate mixer at 1,800 rpm.

3.2.4. Thermotolerance Assay and Fluorescence Data Analysis

Assay Conditions and Data Acquisition

Animals are dispersed in 384-well plates (1 worm per well). Acute thermal stress is induced at 35–37°C, either in an incubator or inside the fluorometer used for detection, for precise temperature control. Fluorescence quantification is performed with a microtiter plate-reading fluorometer (Thermo Labsystems, Beverly, USA). Fluorescence intensity is measured in each well every 30 min, over a 24 h period, with 20 ms integration time for each well. Reading of a 382-well plate is typically completed in about 30 s. The excitation wavelength for the SYTOX Green Dye is 485 nm, with emission wavelength at 538 nm.

Individual fluorescence intensity curves are examined to determine the time at which there is a significant increase in fluorescence over baseline. This point is considered the actual time of death (4). After acquiring all time points of death, a Kaplan–Meier plot can be generated and used to estimate and compare mean lifespan values after specific thermal or other insults (see Subheading 3.1.6 and Chapter 31).

Survival at Specific Time Points After an Insult: Multiple Worms Per Well

1. Dispense bacteria and/or drug-containing media in 384-well plates at a final volume of 55 mL.
2. Add up to 15 sterile adult worms per well (total volume 15 μL), using the automated COPAS Biosorter.
3. Perform stress resistance assays as dictated by the experimental design for each specific stressor.
4. Add SYTOX dye in each well, to a final concentration of 1 μM, a few hours after treatment (to allow death events).
5. Mix for 15 s on a microtiter plate mixer.
6. Incubate at RT for 15 min.
7. Capture images from each well separately using an automated fluorescence microscope (see Note 15). Two images should be captured from each well: one fluorescence image in which only dead worms will be visible (stained with SYTOX) and one bright-field image in which all the animals are visible.

Collected images are analyzed to determine the ratio of the total SYTOX-positive area in each well (fluorescence image), divided by the total area occupied by all worms in the same well (bright-field image). This ratio is indicative of the percentage of survival in each well (5, 9). This analysis can be automated by using the open-source cell image analysis software CellProfiler (5, 15). For additional information on whole-animal, high-throughput analyses based on *C. elegans* see also ref. 9.

3.3. Lifespan Assessment on a Chip: The Microfluidics Approach

3.3.1. The Microfluidic Device

Microfluidic devices are typically fabricated with poly-dimethylsiloxane (PDMS), a biocompatible material, which is mechanically malleable, permeable to O_2 and CO_2, and nontoxic. In addition, it is transparent for wavelengths above 230 nm, allowing bright-field and fluorescence imaging analysis. Directly after sealing the devise on PDMS-coated glass substrate, the walls are coated with 2-(methoxy(polyethyleneoxy)$_{6–9}$propyl) trimethoxysilane (mPEG-silane) to reduce bacterial absorption that would interfere with continuous liquid flow and would obscure the observation of the worms.

The device contains circular chambers (of 1.5 mm diameter) for growing individual worms. Each chamber is linked on one side, to a channel which allows loading of individual worms and the continuous flow of S-complete medium, and from the opposite side, to a conical 7.5 mm-long clamp channel. The width of the clamp wide side is 100 μm, and is proximal to the chamber, allowing an adult worm to enter the clamp. The other side of the clamp is narrow (25 μm), so that animals may be reversibly restricted and immobilized within the clamp. If animal immobilization is not required, an alternative design can be used, where the clamp is replaced by a constant width channel (100 μm). The flow of liquid through each chamber is regulated by a screw valve, located above the channel on the side of each clamp, proximal to the inlet of the device.

3.3.2. Loading Animals into Micro-Chambers

1. Synchronize worms by allowing synchronous gravid adults to lay eggs on NGM agar plates (see Chapter 31).
2. Incubate plates at 24°C for 2 days. At this point, most worms will be at the L4 larval stage.
3. Preload the device with S-complete medium containing OP50 bacteria at a concentration 10^9 cells/mL (using the network outlet of the device).
4. Once the device is saturated with the feeding bacteria suspension in S-complete medium, add worms from the outlet, one at a time.
5. Add a single worm into the network outlet.
6. Connect a syringe (containing bacterial suspension in S-complete medium) to the network outlet via a polyethylene tubing. Note that all the valves need to be open during this procedure.
7. Depress the plunger of the syringe manually. The resulting flow will carry the worm through the network channels into a random chamber.
8. Once the first worm reaches a chamber, close the valve of the corresponding channel, so that the flow of liquids in this chamber is blocked (see Note 16).

9. Continue until all the chambers are filled with L4 larvae (this procedure takes about 1 min/worm).
10. Within 6 h after loading, worms are big enough not to fit in the orifice proximal to the outlet and thus the continuous flow of liquid from the inlet can begin (see Note 17).

3.3.3. Maintenance of Worms in the Device

Feeding of animals while in the device is achieved by a continuous flow of bacterial suspension (from the inlet to the outlet) at a flow rate of 300 μL/h. In total, 25 mL of fresh bacterial suspension in S-complete medium is added to the inlet reservoir each day. To remove any bacterial aggregates, debris or dust particle that could clog the channels of the device, each bacterial suspension is filtered through a 5 μm porous polycarbonate membrane, before loading into the inlet reservoir. The liquid that passes through the device, exits through the outlet and gathers into a waste reservoir.

Over time, bacteria from the inlet reservoir settle and form aggregates at the junction of tubing connecting the reservoir and the inlet of the device. To prevent clogging of device channels, a bypass outlet is built into the device. Every 1–2 days, fresh bacterial suspension is flushed down the inlet tubing and into the inlet of the device and diverted to the bypass outlet (by closing of appropriate valves on the device), thus removing sediment and aggregated bacteria from the inlet of the device. The waste from the bypass outlet is collected into a third reservoir.

The continuous flow of liquid through the chambers also serves to remove eggs laid by the hermaphrodite in the chamber. If aggregates of eggs form that cannot pass through the 25 μm diameter orifice towards the network outlet, eggs may hatch inside the chamber. In this case, L1 larvae are easily removed with the flow and are separated from the mother. Thus, the synchrony of the population is maintained throughout the experiment (see Note 18).

3.3.4. Immobilizing Animals into Clamps

As mentioned above a microfluidic device provides the opportunity to monitor senescent decline in *C. elegans* by monitoring age-related changes of individual worms throughout lifetime. For longitudinal studies, it is important to choose phenotypes that can be monitored noninvasively so that worms are minimally disturbed by the act of observation. To this end, reversible immobilization takes place in the clamp. For example, to immobilize a worm in chamber 1:

1. Close all the valves of chambers 2–16.
2. Reverse the direction of flow from the network outlet to the inlet, by raising the reservoir connected to outlet in a higher position than the inlet reservoir.
3. For as long as the flow is reversed, the worm is immobilized in the clamp and observation can take place.
4. To return worm back in the chamber, just reverse the direction of flow.

4. Notes

1. Caution: Do not flame or autoclave! Store at 4°C in a dark bottle or wrap the bottle in aluminum foil since it is photosensitive.
2. In addition to the OP50-1 bacteria, two *E. coli* strains available at the *Caenorhabditis* Genetics Center (CGC; http://www.cbs.umn.edu/CGC/) are resistant to antibiotics: the OP50-GFP strain which is resistant to Ampicillin (16) and the OP50-NeoR, resistant to neomycin (17). Since, in this assay worms remain in the same well until their death, it is very important to avoid contamination by using antibiotics and exercising good lab practice. To add more antibiotics in the S-complete medium, OP50 bacteria we can be transformed with plasmids that bear the respective selection marker genes. For example, OP50-1 bacteria can be transformed with the empty pL4440 RNAi vector, which confers resistance to ampicillin, which can then be added together with streptomycin and nystatin in the S-complete medium.
3. A bacteria concentration of 100 mg/mL corresponds to about 2×10^{10} cells/mL. The OD_{600} of the suspension should be around 25. Caution: the measurement of the OD_{600} in a spectrophotometer should be performed with a diluted sample (1/50 dilution) so that the measured OD is below 1.0, within the linear range of the spectrophotometer. Otherwise the measurement will not be accurate.
4. Preparation of mixed worm populations involves transferring of five (5) L4 larvae on a freshly seeded Nematode Growth Media (NGM) plate and incubating at 20°C for four (4) days. For detailed information on the preparation and seeding of NGM plates see Chapter 31 (Chapter 31).
5. Caution: Use sterile conditions!
6. After each wash, spin down eggs for 2 min at $1,500 \times g$ at room temperature.
7. To verify that all eggs hatch during the night, drop 20 μL of L1 suspension on a clean NGM agar plate and monitor under a dissecting microscope.
8. The exact time of FUdR addition can vary when the experiment is performed at different temperatures or when strains with shorter or longer post-embryonic developmental periods are used. It must always be added when worms are well into the L4 larval stage.
9. Alternatively one can use gas permeable adhesive seal which allows air exchange, but prevents evaporation.

10. Moving into liquid media is much easier then moving onto solid media.
11. For antimicrobial drug screening studies, a mutant strain more susceptible to infections (*sek-1(km4)*; available at the CGC), is used to increase the sensitivity of the assay.
12. Monitor worm culture progress by removing aliquots and examining under a dissecting microscope. Ideally the percentage of adult worms in the sample should be no more than 25%.
13. When performing screens for anti-microbial substances, OP50 bacteria in the wells may be substituted for microbes causing infection (5, 18). The presence of bacteria in the wells generally increases baseline fluorescence when working with SYTOX Green. For this reason, bacterial concentration in wells should not exceed 5×10^8 cells/mL. It is also helpful to include no-worm control wells to monitor bacteria-specific background fluorescence with time. The medium dispensing step can be automated by using reagent dispenser machines (for example Multidrop Combi reagent dispenser; Thermo Fisher Scientific, Massachusetts, USA).
14. The chemical compounds to be tested can be added at this step. DMSO (Dimethyl sulfoxide) is a common diluent of many chemical compounds. However, DMSO concentration above 0.6% may adversely affect worm lifespan.
15. Versatile imaging devices are available that enable automated image acquisition from multiwall plates. The High-throughput Digital Imager (HiDI 2100; Elegenics, California, USA), is such a devise incorporating two functionally distinct modules: a plate management module and an image acquisition and management system (14).
16. Closing the valve of a chamber occupied by a worm, will stop liquid flow through this chamber, thus preventing entry of additional animals in the same chamber.
17. L4 larvae are particularly convenient for loading the device, as within a few hours (6–12) they grow to become efficiently trapped within the chambers.
18. The probability of death by internal hatching of progeny (bagging) is increased up to 30–35% when worms are grown in liquid cultures (10). These animals are censored during the experiment.

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