

Video Article

The Replica Set Method: A High-throughput Approach to Quantitatively Measure *Caenorhabditis elegans* Lifespan

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Date Published: 6/29/2018

Citation: Cornwell, A.B., Llop, J.R., Salzman, P., Thakar, J., Samuelson, A.V. The Replica Set Method: A High-throughput Approach to Quantitatively Measure *Caenorhabditis elegans* Lifespan. *J. Vis. Exp.* (136), e57819, doi:10.3791/57819 (2018).

Abstract

The Replica Set method is an approach to quantitatively measure lifespan or survival of *Caenorhabditis elegans* nematodes in a high-throughput manner, thus allowing a single investigator to screen more treatments or conditions over the same amount of time without loss of data quality. The method requires common equipment found in most laboratories working with *C. elegans* and is thus simple to adopt. The approach centers on assaying independent samples of a population at each observation point, rather than a single sample over time as with traditional longitudinal methods. Scoring entails adding liquid to the wells of a multi-well plate, which stimulates *C. elegans* to move and facilitates quantifying changes in healthspan. Other major benefits of the Replica Set method include reduced exposure of agar surfaces to airborne contaminants (e.g. mold or fungus), minimal handling of animals, and robustness to sporadic mis-scoring (such as calling an animal as dead when it is still alive). To appropriately analyze and visualize the data from a Replica Set style experiment, a custom software tool was also developed. Current capabilities of the software include plotting of survival curves for both Replica Set and traditional (Kaplan-Meier) experiments, as well as statistical analysis for Replica Set. The protocols provided here describe the traditional experimental approach and the Replica Set method, as well as an overview of the corresponding data analysis.

Video Link

The video component of this article can be found at <https://www.jove.com/video/57819/>

Introduction

One of the most transformative technological advancements towards understanding the genetic basis of aging was the development of feeding-based RNAi in *C. elegans*¹; prior to the experimental use of RNAi, many phenotypes of aging were not genetically tractable. Feeding-based RNAi is achieved through the production of dsRNA within *E. coli* that matches an endogenous *C. elegans* mRNA: IPTG induces bidirectional transcription across an insert of either *C. elegans* cDNA or a portion of an open reading frame within a plasmid². When *C. elegans* feed upon intact *E. coli*, dsRNA produced by bacteria is transported from the lumen into intestinal cells via the SID-2 transmembrane protein³, and then distributed through the rest of the animal via SID-1⁴. Within each cell, exogenous dsRNA is processed by the Dicer complex into siRNA, which interact with a mature mRNA via complementary base pairing to create a new siRNA-mRNA duplex. This duplex is recognized by the RISC complex and cleaved, thereby degrading the endogenous mRNA⁵. Thus, by merely changing the plasmid insert, one can inactivate the function of nearly any gene within the *C. elegans* genome. This discovery led to the creation of several large feeding-based RNAi libraries- collections of transformed *E. coli* stocks that can be combined to achieve coverage of approximately 86% of known *C. elegans* genes^{6,7}.

Since the advancement of feeding-based RNAi, comprehensive screens in *C. elegans* have led to the discovery of more than 900 genes that alter lifespan when inactivated (as evidenced by the RNAi-phenotype associations curated in WormBase), which we refer to as gerogenes. A role for the majority of gerogenes in longevity control was discovered through feeding-based RNAi in just a few seminal reports (see **Figure 1A** and **Supplemental File 1** for details). In some cases, these gerogenes have been identified based on measuring the viability at a single or a few time points, which fails to provide a quantifiable measure of the change in lifespan with RNAi treatment. In other cases, these genes have been quantitatively assessed for changes in lifespan, as well as additional age-associated phenotypes. For instance, we previously identified 159 genes that were necessary for both normal and increased lifespan of animals with decreased insulin/IGF-1 signaling, and quantified changes in healthspan. Of these, 103 gene inactivations result in a progeric phenotype, as loss resulted in one or more signs of premature aging⁸.

While some gerogenes have been associated with 100 or more studies (e.g. *daf-16*, *daf-2*, *sir-2.1*), over 400 gerogenes have 10 or fewer citations (**Figure 1B**, and **Supplemental File 2**). Thus, while comprehensive feeding-based RNAi screens have discovered and cursorily characterized hundreds of putative gerogenes, how these genes function in longevity control, and the genetic interrelationships between these

gene products remain poorly studied. Full longitudinal analysis for age-associated phenotypes is a prerequisite for identifying genetic interactions between gerogenes (e.g. epistatic interactions, asynthetic interactions, etc.). Gaining deeper insight into the genetic interrelationships between gerogenes requires a high-throughput quantitative method, which also leverages the advantages of feeding-based RNAi.

The most common surrogate measure of aging is lifespan. The traditional approach for measuring *C. elegans* mortality tracks the deaths of individual animals over time within a small population sample. A relatively small number of animals are followed over time and periodically are gently prodded with either a platinum wire or eyelash, with movement as an indicator of viability (**Figure 2A**). This method has been widely used, as it provides straightforward, direct measurements of the average and the maximum lifespan. However, this traditional method is time consuming and relatively low-throughput, which limits the number of animals and conditions that can simultaneously be measured in a controlled manner. A recent simulation study found that many *C. elegans* lifespan studies do not assay a large enough number of animals to be able to reliably detect small changes between conditions⁹. Furthermore, this traditional method involves repeatedly handling the same cohort of animals over time, which in turn can introduce contamination, and can damage or kill increasingly fragile, aged animals.

We have developed an alternative "Replica Set" methodology for measuring *C. elegans* lifespan. To this end, a large population of age-synchronized, isogenic animals are divided into a number of small populations (or replicas). Enough replica samples are generated to cover each time point in the planned experiment. At each observation time point, one of the replicas is scored for the number of living, dead and censored animals, then animals within that replicate are discarded. Thus, over the expected lifespan of the population as a whole, a series of independent subpopulations are periodically sampled (**Figure 2B**). In using replica sets there is no repeated prodding of animals and no repeated exposure to potential environmental contamination. The viability observed at the one-time point is completely independent of every other observation, which minimizes handling and increases throughput by at least an order of magnitude. This has allowed us to quantitate changes in lifespan for hundreds of RNAi clones simultaneously^{8,10}.

Here we present detailed protocols for conducting *C. elegans* lifespan via both the Replica Set and traditional methods for scoring *C. elegans* longevity. We demonstrate that similar results are obtained between the methods. We have developed software to assist in the graphical analysis of lifespan data generated through either approach, which we freely provide under a GPL V3 license (See **Table of Materials**). "WormLife" is written in R¹¹, and includes a graphical user interface (GUI) for plotting data, which has been tested in Mac OS and Linux. Lastly, we compare and contrast the limitations of each method and highlight other considerations when choosing between approaches to measure quantitative changes in *C. elegans* lifespan.

Protocol

1. Traditional Method for Scoring *C. elegans* Longevity

1. Preparation of reagents

- Identify genes to be inactivated via feeding-based RNAi. Purchase transformed stocks of HT115 *E. coli*² containing the RNAi clone of interest. Alternatively, subclone the cDNA of the gene of interest into the multicloning site of the L4440 plasmid.
NOTE: HT115 is an RNase III-deficient *E. coli* strain with IPTG-inducible T7 polymerase activity, which is used to prevent degradation of dsRNA within the bacteria. For lifespan studies that do not use feeding-based RNAi, either HT115 or OP50 *E. coli* on standard NGM plates can be used.
- Prepare 3–6 (or more) 6 cm RNAi plates per test condition (**Supplemental File 3** for recipe). Store RNAi plates at 4 °C before seeding with bacteria for up to several months.
- Grow transformed *E. coli* overnight (16–20 h, 37 °C shaking incubator at 180–220 RPM).
NOTE: HT115 *E. coli* is grown in LB with ampicillin (50 mg/mL). Standard OP50 *E. coli* is not antibiotic resistant and is grown without ampicillin. Culture volume depends on the # of plates, but is typically between 8 and 100 mL of LB, depending on the experimental design.
 - Concentrate bacteria by centrifugation at 3,000 x g for 15–20 min in a benchtop centrifuge. Aspirate the supernatant, and re-suspend the pellet at 1/10th starting volume (i.e. 10x) in LB with ampicillin for HT115 (or without ampicillin for standard OP50).
 - Aliquot 200 µL of concentrated 10x bacteria to each 6 cm plate. Prepare 3–4 replicates per test condition, with 2 extra backup plates, to be used in case of contamination. When preparing plates, label them so the experimenter scoring the assay is blind to the experimental conditions, using a color code or similar coding scheme. Ensure that the code is recorded.
 - Allow open plates to dry in a clean environment such as a laminar flow bench until all liquid has been absorbed or allow covered plates to dry on the bench overnight. Monitor plates while drying to ensure that the agar is dry without over-drying.
NOTE: Over-drying the plates leads to cracking of the agar that will cause *C. elegans* to burrow into the agar. Wet the agar surfaces also promote burrowing.
- Store dried plates within a worm box overnight (up to 24 h) at room temperature to allow induction of dsRNA production. After 1 day at RT, store plates at 4 °C for up to 2 weeks in a sealed zip-lock bag (to prevent plates from drying out). Before use, return plates to room temperature within the zip-lock bag to prevent condensation from introducing airborne fungal contaminants.

2. Synchronization of *C. elegans* with hypochlorite treatment

NOTE: See **Supplemental File 3** for M9 and hypochlorite solution recipes.

- Collect gravid adult animals with M9. Use a plugged glass pipette to move to 2x 15 mL tubes.
- Spin tubes for 30" at ~2,000 rpm. Check for a pool of at nematodes at the bottom of the tube. Aspirate the supernatant. Wash two times with M9 solution, repeating spin and aspiration.
- Re-suspend *C. elegans* in 4 mL of M9. Add 2 mL of hypochlorite solution. Immediately vortex for ~3 min, with periodic vigorous shaking. After 3 min, look for a cloud of eggs under a dissecting microscope. Vortex an additional 10–20 s if eggs have not been released after 3 min.

NOTE: Timing the hypochlorite treatment is essential. Eggs within the gravid adults are what survive treatment. After ~3 min the gravid adults break open and the eggs spill out. However, if eggs are in hypochlorite solution too long after release they will die. Conversely, if gravid adults are not left in hypochlorite solution long enough, no eggs will be released.

4. Quickly wash with M9 solution twice as in step 2.1.2.
5. Re-suspend *C. elegans* egg pellet in 3 mL M9 and transfer to a new 15 mL tube. Allow embryos to hatch overnight in 3 mL of M9 solution with rotation at 20 °C.
6. Calculate the density of L1 animals (or embryos) per μL by dropping 10 μL of L1 solution 3x on a 6 cm plate and count the number of L1 animals to calculate the average number of L1s/ μL .

NOTE: L1 animals will settle over time. Therefore, L1 solutions should be periodically mixed.

7. Seed 50 L1 animals per plate. Invert plates and seal with a rubber band. Place plates in a plastic worm box. Seal box in a large zip-lock bag. Move to a 20 °C incubator.

3. Preventing progeny production by the addition of 5-Fluoro-2'-deoxyuridine (FUdR)

1. Grow animals until L4 stage at 20 °C. Check to see whether synchronized animals have developed to L4 approximately 40 h after seeding (step 2.1.7).

NOTE: *C. elegans* developmental time will vary at different temperatures¹² and growth rates of mutant animals for which rates have not been characterized must be empirically tested.

1. Add 160 μL of 50x FUdR to each 6 cm plate with L4 animals.

NOTE: It is critical to add FUdR at the L4 stage. For convenience, make 1,000x stocks of FUdR by dissolving 1 g FUdR into 10 mL ultrapure H₂O. Filter-sterilize stock FUdR with a 0.2 μm filter and a 10 cc syringe. Aliquot ~1 mL of stock into sterile 1.5 mL tubes. Freeze and store at -20 °C.

2. Inspect plates for the presence of male *C. elegans*. Remove all males.

NOTE: Lifespan is typically measured for hermaphrodites and not males. Hermaphrodites that mate live shorter than unmated animals, even in the presence of FUdR¹³. Males are smaller and thinner than hermaphrodites and can be easily identified by a distinctive hooked tail¹⁴.

3. Put box back into the zip-lock bag. Return to 20 °C incubator.

4. Scoring viability

1. Score viability daily by gently touching the animal on the head with a platinum wire or eyelash. Score animals that fail to move as dead and remove them from the plate (**Figure 2A**). Record the number observed dead for each condition for every observation time point.

NOTE: To reduce the risk of scoring bias, the experimenter must remain blind to experimental conditions and similarly must not reference the results from previous time points during scoring.

2. Censor animals that rupture, die from other obvious development defects, or crawl up on the side of the dish: remove these animals and record the number at each observed time point for consideration in the statistical analysis. Additionally, if males are found, remove them and record the number observed.

3. Repeat from step 1.4.1 daily until no animals remain alive.

NOTE: Should the lawn of *E. coli* diminish significantly or fungus begins to grow upon the plate, transfer all remaining animals to a fresh plate with the appropriate RNAi and FUdR.

5. Data analysis- plotting and statistics

1. Input or open recorded observation data in software supporting survival analysis with the non-parametric Kaplan-Meier estimator¹⁵ and log-rank test¹⁶ (See **Supplemental File 3**). Be sure to include censored animals. Do not include males that were observed, if any.

NOTE: Data formats may vary between software, but a common format is to record each individual animal observed on a separate line. The experimental timepoint at which an animal was observed as dead is the "event" time. If censored, the "event" time is the timepoint at which the animal was censored. There is usually a field or checkbox to indicate censored observations.

2. Plot Kaplan-Meier survival curves. Select fewer than six conditions for a single plot if many conditions were assayed to improve readability.

1. Check for data issues that are visually apparent- missing observations, unexpected control results, *etc.* — and address these before statistical analysis.

3. Use the log-rank test function in your software to perform pairwise statistical comparisons. Ensure that any available options for treatment of censored results are set appropriately for right-censored data.

2. Replica Set Method for Scoring *C. elegans* Longevity

NOTE: While the traditional method requires 3–6 plates per condition (see 1.1.2. above), the Replica Set method requires many more (see step 2.2.2 below). The traditional method follows animals on the same plate throughout an experiment (**Figure 2A**). In contrast, with Replica Set animals are only scored once: many identical replicates are set up at the beginning of the experiment so that one replicate is scored at each time point (per trial) (**Figure 2B**).

1. Library setup.

NOTE: Additional detail on handling RNAi clones is covered here, as the Replica Set protocol is amenable to the simultaneous scoring of many RNAi clones, and can be scaled to well over 100 clones at a time. Collections of RNAi clones are preserved as glycerol stocks maintained in a 96-well format plate. Replica Set experiments use 24-well plates. Each well is a different test condition, which may correspond to a collection of RNAi clones, different chemical treatments, animal strains, and so forth.

1. Assemble the layout of the sublibrary for collections of RNAi clones, such that the following conditions are met:

1. Ensure that within a 96-well collection, well A1 is an empty vector negative control.
2. Insert an additional empty vector control randomly within every 24 wells.

3. Split the 96-well plate into blocks of 24 wells, such that each 24-well plate has at one randomly inserted negative control (**Figure 2C**).
4. Insert a positive control randomly within every group of 24-wells (**Figure 2C**).
NOTE: The positive control is dependent on the experimental question. For example, when looking at a collection of RNAi clones that increase lifespan, *daf-2(RNAi)* is frequently included. Conversely, when looking at shortened lifespan, *daf-16(RNAi)* is frequently used.

2. Preparation of replica sets

NOTE: The number of replicates needed is equal to the minimum number of time points (**Figure 2B**). One must know *a posteriori* how long to measure lifespan prior to the start of the experiment, as well as scoring frequency (e.g. a replica set experiment running 40 days with every other day scoring requires preparing a minimum of 20 replica sets for each condition at the start of the experiment). It is advisable to make ~5 extra backup sets (see 2.2.2 and 2.2.2.3 below).

1. To create a fresh stamp of the RNAi sublibrary, inoculate 200 μ L LB+Amp per well in a 96-well format (600 μ L plates) using a 96-pin plate replicator by the following steps:
 1. Sterilize 96 pin plate replicator by sequentially immersing the pins in 50% bleach, ultrapure H₂O, and ethanol. Keep the pins immersed for at least 30 s for the bleach and ethanol steps. After immersion in ethanol, flame the tips briefly. Repeat.
NOTE: Be sure to rinse off all bleach, and do not leave the pins exposed to bleach for extended periods, as bleach can corrode the stainless-steel pins.
 2. Carefully remove adhesive foil cover from frozen 96-well glycerol stock library plate and gently but firmly grind tips of the sterilized plate replicator into the wells of the still frozen glycerol stocks. Inoculate 200 μ L LB + Amp cultures and seal the inoculated plate with a permeable membrane. Allow cultures to grow overnight on the benchtop.
 3. Sterilize the plate replicator as in step 2.1.1, carefully remove the seal from the liquid culture plate after overnight growth and immerse tips of the replicator pins. Apply the tips with even pressure to a rectangular LB amp+tet agar plate, and transfer bacteria from the pins to the plate gently with a small circular motion, ensuring that adequate space is left between adjacent spots. Allow colonies to grow overnight on the agar plate at 37 °C. See **Supplemental File 3** for preparation of LB + Amp/Tet plates.
 4. Prior to use, store agar plate at 4 °C inverted (lid side down), wrapped in paraffin film.
NOTE: Storing colonies lid side up will allow condensation to cross-contaminate RNAi clones! Colonies of *E. coli* can be stored for 2–8 weeks at 4 °C, depending on particular RNAi clones, as RNAi clones retain efficacy in inducing dsRNA after IPTG induction for variable lengths of time. For small collections of RNAi clones, RNAi efficiency should be empirically determined by RT-qPCR to confirm knockdown.
2. Calculate the minimum number of 24-well plates necessary for one trial of the experiment: (# plates per replica set) x (expected # time points). Ensure that a few extra replica sets are included for handling issues such as contamination (**Figure 2B**).
NOTE: The total number of RNAi clones determine the number of 24-well plates to make one set of replicas for each time point (**Figure 2C**).
 1. Prepare 24-well plates, as step 1.1.2 (**Supplemental File 3** for recipe). Label plates when preparing them so the experimenter scoring the assay will be blind to the experimental conditions, using a color code or similar coding scheme.
 2. Inoculate one set of 1.5 mL LB + Amp cultures for every 10 replicas (see 2.2.2). Inoculate cultures in 96 well deep-well plates using the plate replicator (as in 2.1.3) and bacterial colonies from 2.1.4 (**Figure 2C**, left). Seal with breathable membrane, grow 20 h (37 °C) with shaking.
 3. Seed 120 μ L of an overnight culture to each plate using a 6-well multichannel pipette with adjustable tip spacing (**Figure 2C**, right). Dry plates uncovered in a laminar flow hood until all liquid has been absorbed. Do not over-dry. Store plates overnight at room temperature.

3. Synchronization of *C. elegans* using hypochlorite treatment

1. Calculate the minimum number of animals needed for the experiment: minimum # of L1s needed = (15-20 animals/well)(24 wells/plate) (X plates/replica set)(Y replica sets).
NOTE: The Replica Set method requires more animals than the traditional method. One 10 cm plate full of gravid worms can provide 20,000–50,000 L1 animals depending on the density of gravid adults. Similarly, twenty 6 cm plates yield around ~30,000 L1 animals. Plan to prepare an egg preparation that doubles the minimum number of animals needed. If the population in preparation has starved, re-feed or chunk to a new plate and allow at least three generations on food before proceeding^{17,18}. Be sure to freeze back leftover L1s.
2. Follow steps 1.2 to 1.2.6 as in the traditional method to obtain synchronized L1 animals. Seed 15–20 L1 animals into each well using a 6-well adjustable-spacing pipette and reagent reservoir, similar to 1.2.7. Periodically mix L1 solution prevent settling of animals.

4. Prevention of progeny production by the addition of 5-Fluoro-2'-deoxyuridine (FUdR)

1. Follow step 1.3.1. Prepare sterile 50x FUdR stock (see step 1.3.1.1).
2. When animals reach L4, add 25 μ L of 50x FUdR to each well of a 24-well plate using a 6-channel adjustable-spacing pipette.

5. Score viability

1. Remove one set of replicate plates and flood wells with the M9 solution, record total animals per well, and then gently touch non-moving animals on the head with a worm pick (**Figure 2B**). Animals failing to move are scored as dead. Discard scored plates. Record viability daily.
NOTE: Animals that rupture, show gross morphological defects, failed to develop, or crawled up on the side of the well are to be censored by excluding them from both the dead and total values.
 1. Record the number of animals that are censored within each well at each time point separately from the other values.
 2. Do not score wells that do not have food or are contaminated (e.g. fungus or slime); such wells are considered censored. Also, censor a well if all of the animals display morphological or developmental defects. Record the censoring event for this RNAi clone/well at this time point.

3. After scoring a replicate plate, discard it. Continue scoring a new replicate set each day until all animals across all wells are dead. To reduce the risk of scoring bias, the experimenter must remain blind to experimental conditions and similarly must not reference the results from previous time points during scoring.
4. If all animals within a well were dead at a given time point, then after that day's scoring, examine whether all animals have been scored as dead for two consecutive time points for a given well. If so, scoring viability is no longer needed for that well in subsequent time points.
5. Conduct additional independent experiment trials. Track the results of successive trials separately from those in previous trials, with the trial number noted.

3. Graph Data

NOTE: With the Replica Set method a curve fit is applied to approximate the mean and maximum lifespan. The parameters for assessing *C. elegans* mortality fit a logit curve¹⁹. As most survival tools do not support logit curve fitting, a new program was developed for plotting logit curves (for Replica Set); Kaplan-Meier curves (for the traditional method) are also supported.

1. Get started with the software. Download the release zip file for the current version (See **Materials Section**). Extract the zip file to a folder. See the "readme" file in the extracted folder for additional installation instructions.
1. Start the plotting interface. Find the location of the "code" directory in the folder extracted from the zip file (e.g. "/Users/UserName/Desktop/WormLife/code").
 1. Enter the following commands in the R console, substituting for the folder path just identified. Press enter or return after each entered line: 1) `setwd("/Users/UserName/Desktop/WormLife/code")`, 2) `source("plotGUI.R")`, 3) `openMain()`.
 2. Allow time for the interface to load in a new window, bringing up the default screen as shown in **Figure 3A**. Let the R run in the background.
2. **Format data as comma-separated value (CSV) or tab-separated value (TSV) files. Specify columns and names, dependent on the type of analysis. See Supplemental File 4 (Replica Set) and Supplemental File 5 (traditional/Kaplan-Meier) for pre-formatted example data.**

NOTE: Data must be specified in a "long" format, i.e. one row per strain/treatment per time point per trial. CSV files are recommended for best compatibility between platforms.

1. Remove rows corresponding to observations that were skipped or censored. Check for the absence of missing or non-numeric values, as it may result in an error when the data is imported.
2. For traditional Kaplan-Meier style analysis, do not pool data from independent trials- plot them separately. For Replica Set analysis, pool data from multiple trials, and then investigate using the "TrialView" functionality (see 6.3.3.4).
3. **Using the plotting interface**
 1. Load a data file ("import" under the "File" menu) using the dialog box. To open ".csv" files, change the file type in the drop down to ".csv" (the default is ".txt/.tsv"), navigate to the folder with the data file. Here the file is the Replica Set example data (**Supplemental File 4**).

NOTE: Importing a file when a dataset is already open will replace the current dataset.
 2. Select a file to import to start the import wizard, which walks through identifying the columns of the selected file (**Figure S1A** for Replica Set). If the input data corresponds to a replica-set experiment, choose "Logit" for study type.

NOTE: The import workflow is different between Replica Set (**Figure S1A**) and traditional (Kaplan-Meier) (**Figure S1B**).
 3. "Plot data". Select "Add Line" to begin plotting data. (A line corresponds to one set of conditions. There are features to help with experiments where many conditions were tested).
 1. Plot the control conditions- in the case of the example data N2 (WT) with L4440 (empty vector) RNAi are appropriate. Select "Add Line" under the "Graphs" menu to initiate the Add Line wizard: select the condition to plot and the graphical parameters for representing the line.
 1. To add a curve for a different condition manually, repeat the steps in 3.3.3.1
 2. To change color or plot symbol for a line, select "Modify Line" under the "Graph menu".
 3. To remove a line without clearing all lines, select "Delete Line" under the "Graph menu".
 4. To clear all lines in the current plot, select "Clear Line" under the "Graph menu".
 5. To override the automatically selected maximum x-axis (time) value, use "Set X scale" under the "Graph menu".

NOTE: The y-axis (surviving fraction) always displays 100% (top) to 0% (bottom) in increments of 20%. The x-axis is always displayed in increments of 5. Axis labels are not plotted to enable flexibility in labeling after saving plot images.
 2. To save JPEG-format images of the currently displayed plot, use the "Save Plot" option in the "File menu"; only the current plot is saved.
 3. For creating individual plots for many different conditions in one experiment, the software allows defining and plotting a "series".

NOTE: The implementation of the series concept improves efficiency when working with large experiments.

 1. If starting with a blank plot workspace, add lines corresponding to control conditions that are to be consistent across all plots in the series (see 3.3.3.1).
 2. Define the series with "Define Series" in the Graphs menu. Select a condition corresponding to the line to display first in the series; only one can be selected. Choose graphical parameters (line color and plot symbol) for the line, as in 3.3.3.1.
 3. Review the plots for the different test conditions. Switch the display of the "series line" between conditions using the left/right arrow buttons found on the sides of the main plot window as well as in the top menu bar (**Figure 3A-C**).

NOTE: If the selected series line is the same condition as one of the previously added control/reference lines, the new line may appear overlaid.

4. Define the series to make certain other functions available (Save Series Plots and Trialview- see 3.3.3.4 for the latter). After defining a series, use the "Save Series Plots" option from the "File" menu to save individual plot image files for each plot in the series in a new folder.
4. Trialview — visualizing results from independent trials of a Replica Set experiment. If multiple trials were run for one or more of the sample groups, plot the data from the individual trials and the pooled data from all trials separately to evaluate the consistency between independent trials (See **Figure 3D**).
 1. Set up a series as described in 3.3.3.3. The different trials will be plotted for each of the "varying" sample groups in a different image over the respective trials for the specified reference/control samples.
 2. To save the TrialView images, go to "Print TrialViews" in the Data menu; a JPEG image will be output for each plot in the series.

NOTE: The example results in **Figure 3D** is for a case with two trials.

4. Median lifespan summary table for Replica Set data. Visually inspect curves to ensure reasonable fit of the data, then select the "Summary Table" option in the Data menu to save a table of median lifespan values (time at 50% survival on logit curve) for each sample type.

4. Statistical evaluation of data generated via the Replica Set method

1. For statistical analysis between two groups from a Replica Set experiment, modify and run an R script, provided in the release zip file (see "WormLife statistical analysis template script.R").

NOTE: Proficiency in R is not required to run the script. Additional documentation is in comments in the file. The script is ready for analysis of the example Replica Set data. User-generated data in the appropriate format (see **Supplemental File 4**) can also be analyzed.

1. Open the script file in R (R GUI or RStudio).
NOTE: This will display the script without running it.
2. Modify the locations of necessary files to match the location on your computer.
NOTE: These file paths must be "fully qualified" (i.e. should include the complete file location, including folders).
 1. Modify the paths (file/folder locations) for "wDir" (location of the directory), "dataFile" (the Replica Set data file to analyze), "compFile" (specification of comparisons to run, if applicable), and "outputPath" (location where result files will be written to).
3. Set the column names in the "Specify columns in data file" section if column names in the file to be analyzed are different from the example file. Specify a column for each parameter. If a study has multiple strains, but no RNAi (or other treatment) include a column in the data file with blank entries or the same for all rows (e.g. "no_treatment", etc.).
4. Set the "compFile" parameter. If the "compFile" parameter is set to NA, then all possible pairwise comparisons of groups will be run.
NOTE: A group is defined by the combination of strain/genotype and treatment, which are concatenated and displayed as "strain_treatment". For larger studies with many groups, a CSV file specifying comparisons can be provided. See the example file "comps_rsm_example.csv".
5. Set the number of iterations for Monte Carlo resampling ("k.resamp", the default is 1,000).
NOTE: P-values of 0 may be returned when too few iterations are performed; in such cases it is appropriate to report "p < 0.01" (if k.resamp = 100), or "p < 0.001" (if k.resamp = 1,000), etc.
6. Run the script: "source" button in RStudio (top right of the edit window) or "source document" in the "edit" menu in the R GUI. (Execution may take some time.)
7. When the R "busy" indicator is no longer displayed, open the output directory- there will be a table with results of each comparison (median survival, p-values, % median lifespan change).

Representative Results

In the development of any new methodology, it is imperative that the new method recapitulates accepted results from previous approaches and meets the standard within a field. We have previously shown empirically that the Replica Set and traditional methods for assaying *C. elegans* lifespan produce similar results²⁰. Wild-type *C. elegans* (N2) maintained at 20 °C typically live between 20 and 25 days, which we observed with both the traditional (**Figure 4A**, black line) and Replica Set approach (**Figure 4B**, black). Thus, both methods reasonably approximate wild-type lifespan. It is also essential that a new method has the resolution to accurately quantify changes between test conditions and the statistical power to detect significant changes. In our previous study, we discovered that the Myc family of transcription factors are determinants of *C. elegans* longevity. *mml-1* and *mxl-2* encode the *C. elegans* homologs of mammalian Mondo A/Carbohydrate Response element binding protein (ChREBP) and Mlx, respectively. In both *C. elegans* and mammals, these Myc-family members heterodimerize to regulate transcription. We found that loss of either *mml-1* or *mxl-2* significantly decreases normal lifespan, as measured by either a traditional lifespan assay or by Replica Set (**Figure 4A-B**, yellow and maroon). In contrast to the MML-1::MXL-2 complex, we found that loss of either *mdl-1* (homologous to mammalian Mad) or *mxl-1* (Max) significantly increased *C. elegans* lifespan as measured by either methodology (**Figure 4** purple and blue, respectively, in both panels).

A serious limitation to the traditional approach for measuring longevity is throughput. Both methods rely on movement to call whether an animal is alive or dead, which becomes increasingly difficult to assess. Young animals will move throughout a plate in the absence of stimuli and are thus easy to score. Aging *C. elegans* become increasingly sedentary but will respond to a light touch to the head by a reversal movement on a plate. However, as animals become older the ability to move backward diminishes and becomes increasingly uncoordinated. Ultimately animals become paralyzed, a phenotype strongly resembling sarcopenia, and when scoring via the traditional method viability can only be determined by observing subtle twitch at the extreme anterior tip of the animal. In contrast, when scoring viability via the Replica Set method, the liquid is added to the well, which acts as a stimulus that generates a thrashing response that can be quantified as a readout of healthspan⁸. Movement in the liquid is easier to observe for even older animals: chronologically age-matched decrepit animals produce subtle head movements on dry plates but a more pronounced (albeit slow) body bend in liquid. Finally, when scoring Replica Set, the whole well is within the field of view (~1.1 cm in diameter) and all animals are in suspension- allowing observation of all animals simultaneously. In contrast, when scoring a 6 cm plate via the traditional method, one must scan across the entire plate -searching through the bacterial lawn and along the edges for animals. The net consequence of these differences is that the throughput when using the Replica Set method is at least an order of magnitude greater than the traditional approach, which makes it possible to simultaneously quantify changes in lifespan across more than 100 conditions in a single experiment with one investigator. For example, from a genome-wide feeding-based RNAi screen we previously identified 159 genes that were necessary for the increased lifespan conferred by decreased *daf-2*/insulin-like signaling⁸. In that analysis, we quantified the changes in lifespan in wild-type, a long-lived *daf-2(e1370)* mutant, and short-lived *daf-2(e1370);daf-16(mgDf47)* double mutant animals (**Figure 5A**), which allowed us to decipher the genetic relationships between insulin-like signaling and over 100 progeric gene inactivations. Further, we assessed how these progeric gene inactivations altered healthspan (at the time called "activespan") by observing the decline in *C. elegans* thrashing across replicates over time (**Figure 5B**).

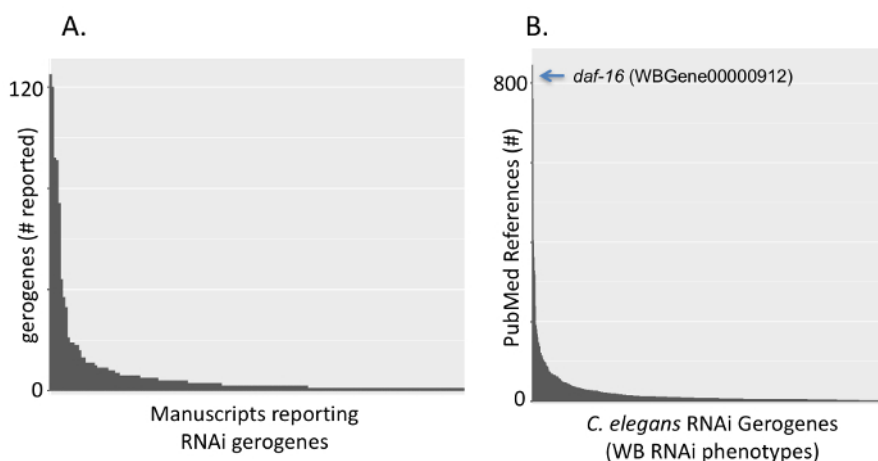
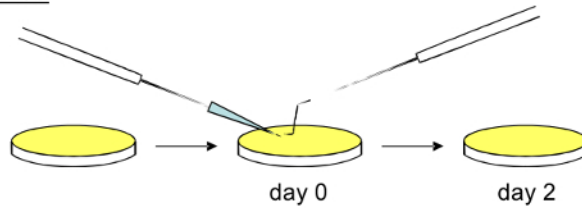


Figure 1: The advent of feeding based RNAi lead to an era of gene discovery in aging research, yet most gerogenes remain poorly studied. (A) Many gerogenes were initially discovered from large scale functional genomic screens. Of the more than 900 *C. elegans* gerogenes discovered to date, many were identified using feeding-based RNAi, highlighting the value of functional genomic approaches in gene discovery. The graph shows the number of gerogenes discovered per manuscript using RNAi, based on phenotype annotation (See **Table of Materials**) for phenotype ontology terms extended life span, shortened life span, and life span variant. See **Supplemental File 1** for the full list of studies that discovered gerogenes. **(B)** Most gerogenes remain poorly studied. In contrast to well-studied gerogenes like *daf-16*/FOXO (arrow), which has more than 800 references, the majority of gerogenes have fewer than 10 references (general reference- not necessarily focused on lifespan). Reliable high-throughput methods will be essential to derive deeper insight into the genetic inter-relationships between gerogenes. The graph is based on mappings between publications in PubMed and the *C.elegans* gerogenes discovered from RNAi phenotypes. See **Supplemental File 2** for the full list of gerogenes and number of studies associated with each. [Please click here to view a larger version of this figure.](#)

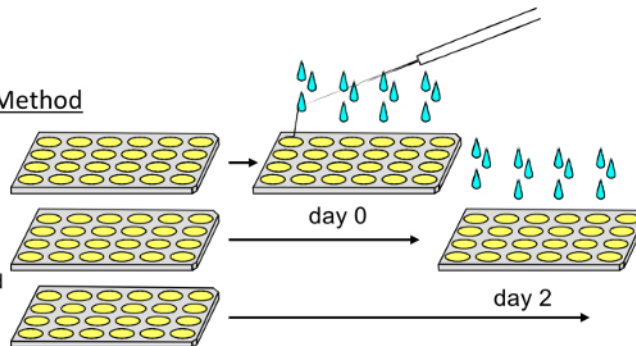
A. Traditional Method

- Limited # of sets/condition
- Same set followed over time
- Viability assessed by physical stimulus (poking)



B. Replica Set Method

- Many sets per condition
- Each set scored once
- Viability assessed by adding liquid and poking



C. 96-well *E. coli* liquid culture

24-well RNAi plate

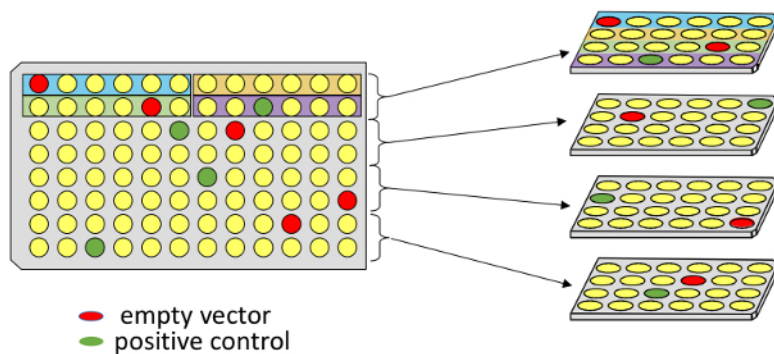


Figure 2: The Traditional and the Replica Set Method for scoring *C.elegans* lifespan (A). The Traditional Method for scoring *C. elegans* lifespan. Several small synchronized populations of isogenic animals per condition are followed over time. The same population of animals is followed throughout the study course. Viability is assessed by movement, which may be stimulated by gentle prodding. Animals that fail to move are scored as dead and are removed (aspiration shown) until no viable animals remain. **(B).** The Replica Set Method for scoring *C. elegans* lifespan. A large population of age-synchronized isogenic animals are distributed across a number of identical replicate plates. At each time point, a single replicate is scored: a mild buffered solution (M9) is added, which stimulates movement. Animals that fail to move spontaneously after flooding wells are also assessed via touch stimulus. The scoring duration for the experiment is determined prior to the start. Each animal is scored only once and longevity for the larger population is derived from many independent observations. **(C).** The Replica Set approach is a high throughput method to quantitatively measure *C. elegans* lifespan. 100 or more independent RNAi clones can be tracked simultaneously. HT115 *E. coli* expressing dsRNA for a given RNAi clone is shown. Practically, every 24 samples from the 96-well plate are divided into a single 24-well plate. Each resulting 24-well plate has a negative (*i.e.* empty vector, red well) and positive control (green well) randomly distributed within a collection of RNAi clones (yellow wells). Typically, the first well (A1) in a collection contains an empty vector. [Please click here to view a larger version of this figure.](#)

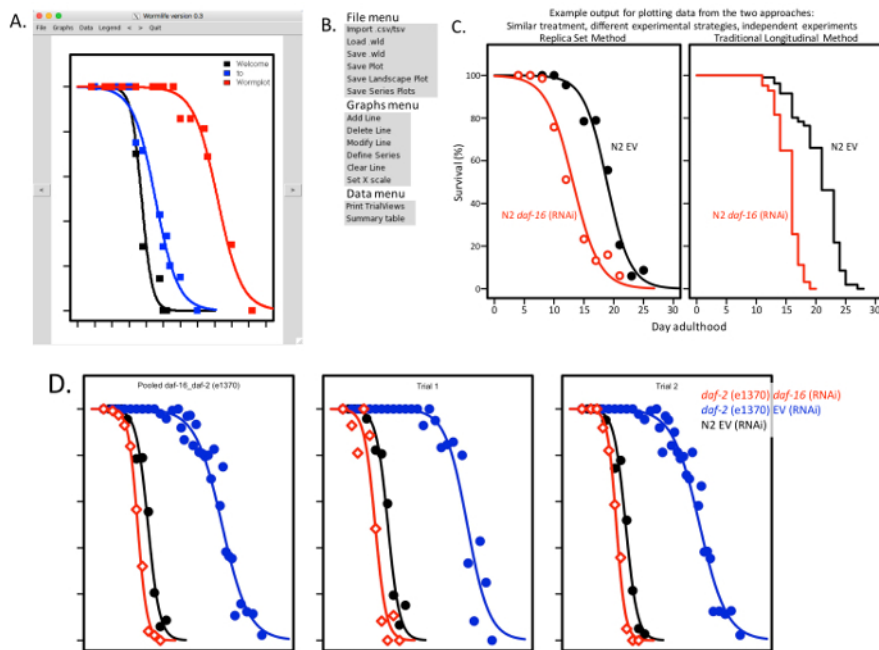


Figure 3: The graphical user interface (GUI). (A). The main plot window interface showing the default welcome screen. This is what is displayed upon opening the software. Differences in appearance between platforms should be minimal due to use of a platform-independent windowing toolkit. (B). Overview of menu options, for the drop-down menus available from the main plot window. (C). Example plot output for both Replica Set style (left) and traditional Kaplan-Meier style (right) data. The data displayed was collected in independent experiments. Exported plots do not include pre-plotted axis labels, for maximum flexibility in adding such labels. To facilitate this, the axes are always divided into increments of 20% for the Y-axis, and increments of 5 for the X-axis. In this example, axis and line labels (strain/treatment) were added to the saved plots, using a very simple and common image editing tool. (D). An example of output from the "TrialView" functionality, allowing for the visual comparison between results of independent trials for Replica Set style datasets. This plot shows the result between two different trials and the corresponding pooled results for *daf-2* EV(RNAi) (blue, closed circles), N2 EV (RNAi) (black, closed circles), and *daf-2* with *daf-16* (RNAi) (red, open diamonds). TrialView allows for quickly checking for trial-specific data issues that might affect the quality of the fit of the pooled dataset. [Please click here to view a larger version of this figure.](#)

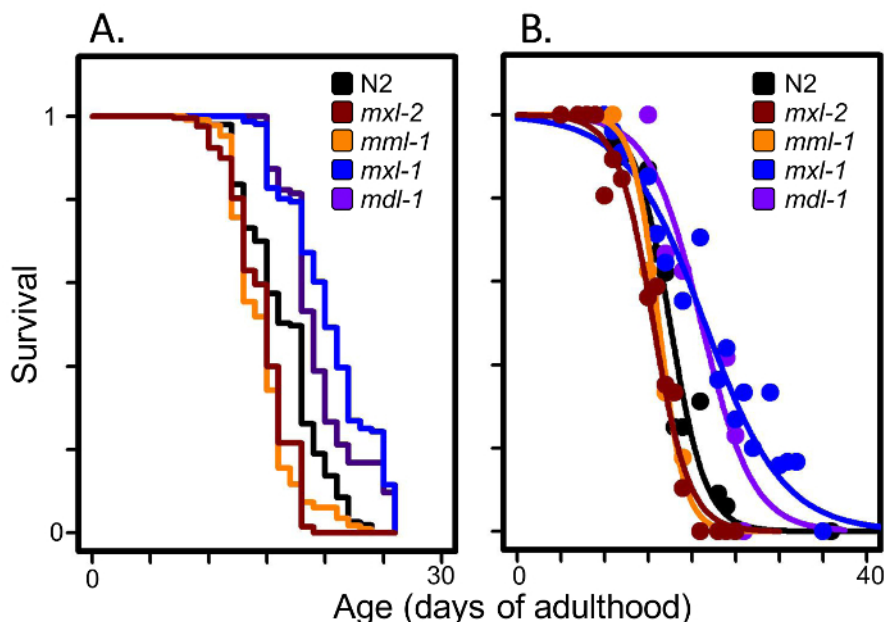


Figure 4: The Traditional and Replica Set methods produce similar results. Loss of either component of the MDL-1(Mad)::MXL-1(Max) heterodimer increases lifespan. In contrast, loss of either component of the MML-1 (Mondo/ChREBP)::MXL-2(Mix) decreases lifespan. This figure is reprinted from reference²⁰ with permission via a Creative Commons Attribution (CC BY) license (See Materials). (A). Kaplan-Meier results with the traditional method. (B). Logit curve fit using the Replica Set method. [Please click here to view a larger version of this figure.](#)

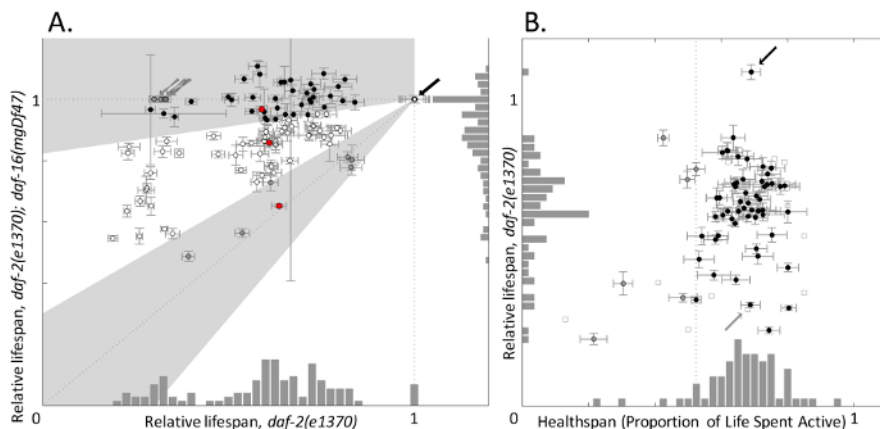


Figure 5: The Replica Set method can decipher genetic interactions based on changes in lifespan (A) and alterations in healthspan (B) for over 100 RNAi clones simultaneously. This figure is reprinted from⁸ with permission under the Creative Commons Attribution-Non-Commercial 4.0 International License (CC-BY-NC) (See Materials). (A) Genetic lifespan analysis of progeric gene inactivations in the context of decreased insulin-like signaling (ILS) (*daf-2*, x-axis) and in the absence of *daf-16*/FoxO (Y-axis), a central transcriptional effector of ILS²¹. Gene inactivations with similar functions as *daf-16* do not further shorten lifespan in the absence of *daf-16* (black dots). Gene inactivations with functions completely independent from *daf-16* shorten both genetic backgrounds similarly (grey). Gene inactivations where the negative effect on lifespan in *daf-2* > *daf-16*, suggests function in parallel (white). (B) Changes in thrashing rates over time can derive the average healthspan for many genetic perturbations (x-axis) while assessing changes in lifespan (y-axis). [Please click here to view a larger version of this figure.](#)

Figure S1: Workflows in WormLife. Illustration of the steps of some guided workflows (sometimes termed "wizards"). In each of these cases, after the last step in the workflow, the focus is returned back to the main plot window. (A) The data import workflow for Replica Set style datasets (B) The data import workflow for traditional Kaplan Meier style datasets. (C) The workflow for adding lines to a plot for Replica Set style datasets. (D) The workflow for adding lines to a plot for traditional Kaplan Meier style datasets. [Please click here to download this file.](#)

Supplemental File 1: Studies that have identified gerogenes. The advent of feeding-based RNAi led to an era of gene discovery for phenotypes that were not tractable by forward genetics, including aging. Listed, in the order of the number of gerogenes discovered, are independent studies that identified genes whose activity altered lifespan. Note that the study that identified the most gerogenes utilized the replicate set method⁸. The nature of how gene inactivations altered lifespan is also indicated: longevity and progeric genes are those that increased or decreased lifespan when inactivated, respectively. "Life span variant" refers to cases where directionality of change (*i.e.* increased or decreased lifespan) was not specified or has not been curated. Data from WormBase WS262 (January 2018) (See Materials), with the addition of RNAi-treatment lifespan results from reference¹⁰, which are not yet included in the curated collection of WormBase RNAi phenotypes. [Please click here to download this file.](#)

Supplemental File 2: The number of studies associated with each gerogene. Most gerogenes are poorly studied. While some genes, like *daf-16*/FoxO, have been the subject of much research attention, more than 400 gerogenes have fewer than 10 associated publications. Data from WormBase WS262 (January 2018), with the addition of RNAi-treatment lifespan results from¹⁰ which are not yet included in the curated collection of WormBase RNAi phenotypes. [Please click here to download this file.](#)

Supplemental File 3: Preparation of common reagents for *C. elegans* experiments. (A). Recipe for standard NGM and RNAi plates. (B). Recipe for M9 buffer and hypochlorite solution. (C). Preparation of LB +Amp/Tet plates. [Please click here to download this file.](#)

Supplemental File 4: Replica Set example data. An example dataset from a Replica Set lifespan experiment. This dataset is already formatted to be suitable for import/analysis. Includes two trials per condition (combination of strain/genotype and RNAi). [Please click here to download this file.](#)

Supplemental File 5: Traditional longitudinal example data. An example dataset from a traditional longitudinal lifespan experiment, with right-censoring, formatted for ready import/analysis using Kaplan-Meier survival plot functionality. [Please click here to download this file.](#)

Discussion

Both the traditional and replica set methods require the synchronization of chronologically aged animals. We include a method that synchronizes animals using hypochlorite treatment of gravid adults, where only fertilized eggs with the gravid adult survive treatment. These embryos hatch in liquid suspension and developmentally arrest at the first larval stage (L1). After seeding L1 animals onto food (*e.g.* *E. coli* expressing dsRNA to a gene of interest), animals resume development. Synchronizing L1 animals by hypochlorite treatment of gravid adults has the advantage that the leftover unseeded L1 animals can be frozen and stored indefinitely in liquid nitrogen or a -80 °C freezer. In this way, a sample of each strain at the time of the experimental setup is preserved, creating a valuable resource for future studies and improving reproducibility. However, while hypochlorite treatment of gravid adult animals is a common way to obtain synchronized animals for aging research²², the L1 arrest is a starvation response. Thus, some laboratories prefer either to allow hatching to occur on plates, or to forgo hypochlorite treatment altogether and allow a few gravid adults to lay eggs for several hours (*i.e.* an egg lay). In the latter case, parents are removed and the progeny lifespan is followed. To the best of our knowledge, no obvious differences in lifespan have been reported between animals that were synchronized in M9, hatched on plates, or progeny from an egg lay. However, given that changes in nutrient availability are closely linked to changes in lifespan, there

is precedence that specific genetic backgrounds could produce different outcomes between these synchronization approaches. A more careful analysis is required to resolve this theoretical concern.

Regardless of the method used to synchronize the starting population, steps must be taken to either prevent progeny production or to separate the synchronized starting population from future progeny. In our protocol, we outline how to use FUDR to prevent progeny production, as separating animals is not a viable option for the replica set method. It is also possible to prevent progeny production genetically through the use of a feminized genetic background (e.g. *fer-15(b26);fem-1(hc17)*, which is a temperature-dependent sterile strain²³). However, neither is without shortcomings: the use of genetic backgrounds can complicate subsequent analysis, and in some genetic backgrounds FUDR can alter longevity^{24,25,26}.

As an alternative to preventing progeny production through chemical or genetic means, adult animals can be periodically moved to fresh RNAi plates to isolate them from their progeny. This simplifies background considerations at the expense of throughput. Periodically moving animals to fresh food has the additional advantages of preventing possible starvation and renewing exposure to dsRNA. However, some genetic interactions that influence lifespan were only discovered when progeny production was inhibited: early analysis of the TGF β pathway for a lifespan phenotype erroneously concluded that decreased TGF β signaling influenced *C. elegans* dauer formation but not aging^{27,28}. However, a follow up study that used FUDR revealed that decreased TGF β signaling increased longevity through insulin signaling²⁹. Why did earlier studies fail to see increased lifespan in TGF β mutant animals? TGF β pathway mutations produce a slight egg laying defect (*egl*) and extend reproductive longevity, which causes internal hatching of progeny later in life that kills the parent. It is likely that the long-lived TGF β mutant animals appeared to have a normal lifespan because of the *egl* phenotype killed the animals around the time when wild-type animals normally die. This might be relevant to other genetic pathways linked to DR, as starved wild-type animals also manifest an *egl* phenotype, perhaps as an adaptive survival advantage to progeny under conditions of low food. This highlights the underlying complexity in adaptive responses animals undergo under stress conditions, and the need for careful analysis and consideration when designing lifespan experiments.

In designing and conducting lifespan experiments by either method it is critical to avoid bias. Experiments must be conducted in a double-blind manner: how samples were previously scored at previous time points and the identity of a test condition must be unknown to the experimenter. Furthermore, it is always necessary to include both positive and negative controls; in the case of the replica set method, these are randomly inserted into a 24-well plate. *E. coli* expressing a plasmid that does not contain an insert with the sequence corresponding to the *C. elegans* genome is the empty vector negative control (i.e. "L4440"- See Materials). Positive controls are dependent on the specific nature of an experiment. For instance, *daf-2* encodes the *C. elegans* insulin/IGF-1 receptor, and *daf-2* inactivation via feeding-based RNAi robustly increases lifespan at least two-fold in wild-type animals²⁷. Thus *daf-2(RNAi)* might serve as a positive control when looking for gene inactivations that increase lifespan. Conversely, *daf-16* encodes the FOXO transcription factor orthologue³⁰. DAF-16 is an essential component of many longevity paradigms and wild-type animals (N2) treated with *daf-16(RNAi)* are short lived and show signs of progeria³¹.

The primary advantages of the traditional longitudinal lifespan approach are that it is very well established, and experiments are easy to set up. Relatively few animals are needed on just a few plates for each test condition. Thus, strains that grow poorly or require balancers to propagate can be easily tested. The traditional approach is highly adaptable and can be used with any one of the available approaches for handling progeny production, including treatment with FUDR, crossing into a feminized genetic background, or periodically moving adult animals to new plates during the egg-laying period. While moving animals greatly reduces throughput, working with a mutant background is never ideal, and although FUDR does not alter wild-type lifespan^{32,33,34}, it can affect lifespan and age related phenotypes in some genetic backgrounds^{35,25,26}. Note that the presence of males, even with the use of FUDR, will significantly shorten hermaphrodite lifespan¹³, thus a plate that contained males after the hermaphrodites reached L4 is unusable. Likewise, analysis through the Kaplan-Meier estimator and associated curves, and the log-rank test, is well established for mortality data. However, there are several disadvantages to the traditional lifespan analysis. Repeated handling of plates (i.e. exposing the plates to air) facilitates the introduction of airborne fungal contamination. Additionally, repeated poking can damage or kill animals, especially as the population advances in age and become fragile. Older animals become largely paralyzed and mired in *E. coli*, while *E. coli* becomes an opportunistic pathogen (colonizing the lumen and packing the pharynx)³⁶. Very old living animals can only be identified by subtle head movements. Thus, it is easy to classify a decrepit live animal as dead. Lastly, the traditional approach is limited by throughput.

The Replica Set method is high throughput and quantitative. However, the disadvantage of this method is a larger investment of time and resources in the initial set up. A moderately large experiment to examine 100 RNAi clones over 20 time points requires 30,000 L1 animals (where approximately 15 animals are examined per RNAi clone per time point), which while easy for most strains, can be problematic in some cases. For instance, without a large-particle sorter ("worm sorter") strains that must be maintained with balancers or transgenic lines carrying a poorly transmitted extra-chromosomal array cannot be easily examined by this method. A second disadvantage is that progeny production must be inhibited, which requires the use of FUDR or a feminized genetic background. Finally, one must know the length of time the assay will run, as one must prepare a replica set for each time point at the beginning of the experiment. However, the advantages of this method are numerous. Foremost, scoring viability is much faster and one can easily follow animals over 100 test conditions simultaneously (i.e. RNAi clones). Since a replica set is only scored once then discarded, there is no repeated handling of plates or poking of animals, which minimizes the likelihood of fungal contamination and eliminates mortality caused by the occasional rough prodding with a worm pick. Furthermore, the addition of liquid to the well greatly facilitates scoring. Freeing old animals from the plate and surrounding bacteria assists in allowing subtle head movements to be more easily scored. Addition of liquid also provides the opportunity to measure thrashing rates as a measure of fitness (e.g. healthspan^{8,37}).

Aging is a complex phenomenon involving multiple causal mechanisms which require use of systems biological approaches to unravel. These approaches often incorporate data-driven modeling, using large volumes of genomic/transcriptomic data, and require complementary robust and high-throughput methods to measure lifespan and healthspan. The high-throughput Replica Set method will allow comparison of many RNAi clones longitudinally, while minimizing batch effects and technical errors, thus facilitating the development of dynamic models that can infer the interactions between causal pathways in a quantitative manner. Additionally, integration of several genome-wide genomics approaches with the Replica Set method is feasible because a large population of age-synchronized animals is divided into a number of small populations.

Other methods have been previously developed to improve the throughput of *C. elegans* lifespan experiments, often focusing on adapting the traditional longitudinal approach (i.e. following the same set of animals over time) to automated observation and recording using common flatbed scanners^{38,39}, or more specialized equipment such as microfluidic plates⁴⁰. The scanner-based approaches use light as a stimulus and compare sequentially captured images to determine alive/dead status based on movement for multiple plates at one time; while such approaches do not

require proprietary scientific hardware, time involved in setting up the workflows may be substantial depending on the desired scale. Alternatively, lifespan experiments in custom microfluidic devices allow for in-depth phenotypic characterization of single animals over time, and without treatment to prevent progeny, but necessitate fabrication of the microfluidic plates and acquisition of associated microfluidic pumps and imaging equipment. In contrast, the Replica Set method, in combination with the software detailed here, allows greatly improved throughput using tools that are already common in laboratories working with *C. elegans*.

The WormLife software will be improved in the future to offer easier access to the statistical comparison, and compatibility with additional platforms. The most up-to-date documentation for the software can be found at the GitHub page, including installation instructions for platforms on which the software has been tested. A web-based version will also be developed to enable convenient access without the need to install any software.

In summary, the combination of the Replica Set method and the freely available software detailed here provides a powerful platform for improving the throughput and robustness of lifespan experiments and a broad range of survival-based assays (e.g. stress tolerance, toxicology studies, healthspan, etc.). Particularly when combined with functional genomics, this approach leverages the many benefits of the metazoan model system *C. elegans* for deciphering the myriad of genetic interactions that contribute to the progression of aging.

Disclosures

The authors declare that they have no competing financial interests.

Acknowledgements

Funding for this work described in this manuscript was provided by: the University of Rochester Office of The Provost and the School of Medicine and Dentistry Dean's Office via the Health Sciences Center for Computational Innovation (HSCCI); the Ellison Medical Foundation New Scholars in Aging Fellowship (AG-NS-0681-10) The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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