# *Ce*Lab, a Microfluidic Platform for the Study of Life History Traits, reveals Metformin and SGK-1 regulation of Longevity and Reproductive Span

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## Abstract

The potential to carry out high-throughput assays in a whole organism in a small space is one of the benefits of *C. elegans*, but worm assays often require a large sample size with frequent physical manipulations, rendering them highly labor-intensive. Microfluidic assays have been designed with specific questions in mind, such as analysis of behavior, embryonic development, lifespan, and motility. While these devices have many advantages, current technologies to automate worm experiments have several limitations that prevent widespread adoption, and most do not allow analyses of reproduction-linked traits. We developed a miniature *C. elegans* lab-on-a-chip device, *Ce*Lab, a reusable, multi-layer device with 200 separate incubation arenas that allows progeny removal, to automate a variety of worm assays on both individual and population levels. *Ce*Lab enables high-throughput simultaneous analysis of lifespan, reproductive span, and progeny production, refuting assumptions about the Disposable Soma hypothesis. Because *Ce*Lab chambers require small volumes, the chip is ideal for drug screens; we found that drugs previously shown to increase lifespan also increase reproductive span, and we discovered that low-dose metformin increases both. *Ce*Lab reduces the limitations of escaping and matricide that typically limit plate assays, revealing that feeding with heat-killed bacteria greatly extends lifespan and reproductive span of mated animals. *Ce*Lab allows tracking of life history traits of individuals, which revealed that the nutrient-sensing mTOR pathway mutant, *sgk-1*, reproduces nearly until its death. These findings would not have been possible to make in standard plate assays, in low-throughput assays, or in normal population assays.

## Introduction

*C. elegans* is a useful model system to study a variety of biological characteristics, but it is particularly well-suited to the study of aging because of its short lifespan and obvious, visible changes with age. Its genetic tractability, short generation time (<3 days), large progeny number (300 if self-fertilized, >600 if mated) and high degree of evolutionary conservation has allowed the discovery of conserved genetic regulators of development, longevity, reproductive aging, health span, and other important life history phenomena. Its small size (<1mm) and simple laboratory diet (*E. coli*) enables the maintenance of large numbers of animals in a small area. Together, these properties make *C. elegans* ideal for high-throughput experimentation and discovery applications, particularly for life history traits such as lifespan, progeny production, and reproductive span.

*C. elegans* assays often require a large sample size and frequent physical manipulations, rendering them highly labor-intensive[1]. Some of these challenges have been met by long-term culturing and imaging[2,3] that have been used for large-scale lifespan analyses[4–6]. For example, a recent high-throughput analysis of lifespan and motility traits[7] re-emphasized that induced Maximum Velocity best reflects health of individuals with age, as we previously reported[8]. However, such large-scale approaches usually require the elimination of progeny, limiting the analysis to lifespan and healthspan metrics that ignore reproductive traits and reproductive effects on lifespan. Therefore, other approaches are necessary to assess healthspan parameters associated with reproduction, such as progeny production, reproductive span, and post-mating lifespan[9,10]. Preparing growth medium in well plates for large-scale cultivation can potentially be a viable alternative, as it offers flexibility in experimental design for users to simultaneously test various biological conditions. However, in smaller wells, worms are easily lost to desiccation on the walls. Moreover, users are still required to carry out laborious transfer of worms. To address these problems, liquid assays in well-plates can expedite daily handling. However, worms in liquid alternate between stressful swimming and long quiescence states[11,12] and they may also experience hypoxic conditions that compromise long-term incubation.

Microfluidic technologies have emerged as state-of-the-art tools to expedite worm assays, which can also offer a higher degree of control and accuracy. The integration of novel lab-on-chip devices with programmable valves, motorized platforms, image-based screening

methods, and computer-assisted analysis has enabled faster development of new disease models and rapid phenotypic screening of large drug libraries[13]. Pioneering microfluidic chips have been designed for worm culture[14], embryonic developmental studies[15,16], microsurgery[17], behavioral screening[18], and high-content imaging[19,20]. Although advanced, these technologies are often assay-specific and single-use, and have limited capacity and flexibility in experimental design (Table S1). Moreover, they are often complicated to operate and slower than traditional assays, as new devices or chips are required for each experiment. Despite excellent proof-of-concept work, these drawbacks have prevented the widespread adoption of this technology.

Here we describe our development of *Ce*Lab, a microfluidic device for the study of *C. elegans* life history traits. Because *Ce*Lab solves problems of previous devices and plates, we were able to use it to discover new aspects of biology, as well. To automate multiple manual assays using one device, we have developed a reusable device that combines features of multi-well plates, microfluidic chips, and manual assays to address the limitations of previous technologies while retaining their best qualities. *Ce*Lab is a plug-and-play device that uses novel techniques for worm loading and operation of the device to close the gap between engineering design of microstructures and macro-world of conventional worm assays. Our device is highly reliable, versatile, and easy to use, with minimal operational costs and environmental impact, and offers high flexibility in experimental design. With daily bacterial feeding and daily manual scoring on a benchtop microscope, it closely resembles plate assays in its output, but offers individual worm information that is not possible in population plate assays. We found that carrying out lifespan and reproductive span assays using *Ce*Lab is ~6x faster than corresponding plate assays (Table 1) which can significantly expedite worm research, enabling new biological discoveries. *Ce*Lab allowed us to discover that individual animals are not subject to the Disposable Soma hypothesis of resource utilization, and that heat-killed bacteria extend lifespan and reproductive span through specific genetic mechanisms. Finally, we found that mutation of *sgk-1*, the Serum- and Glucocorticoid-inducible kinase homolog, greatly extends reproductive span and reverses lifespan shortening normally observed after mating. These biological observations were possible because *Ce*Lab enables high-throughput population and individual analyses of multiple healthspan characteristics in reproductive animals simultaneously.

## Results

We are interested in the high-throughput measurement of lifespan, reproductive span, progeny production, and health metrics of individual animals, all of which requires long-term automated monitoring of individuals, daily removal of progeny, easy loading of many animals, and visualization of health span characteristics that are comparable to those seen on plates with manual manipulation. To meet these requirements, we needed to develop a new device.

**Design and use of *Ce*Lab.** We designed and fabricated a multilayered Polydimethylsiloxane (PDMS) device for long-term *C. elegans* incubation and monitoring (Figure 1a-g, Figure S1a-d). One of the first requirements for long-term maintenance of *C. elegans* in microfluidic chambers is the presence of pillars[21] (Figure 1a), as thrashing worms in pillarless environments are stressed and have shorter lifespans[14]. Hexagonally-arranged microposts allow animals to move in the same fashion as they do on an agar plate[18] (Figure 1a). Imaging of the worms inside the chamber (Figure 1d) enables measurements of size and health span.

Several aspects of long-term maintenance require daily buffer exchange: worms require fresh food (*E. coli*) and removal of potentially toxic metabolites and old bacteria daily (Figure 1b, c). 200 chambers, each housing an individual worm, each have outlets for loading and daily buffer exchange (Figure 1d). Manual assays require laborious daily transferring of tested populations to new plates to avoid progeny interference with assays (Table 1) or sterilization of adult worms through a variety of methods (fluorodeoxyuridine (FUdR) or blocking of self-sperm development)[22]. However, these progeny-blocking interventions may affect growth, development, aging, or interfere with chemical screening[14,23]. Instead, *Ce*Lab assays of reproductive worms uses flushing of progeny daily. Capable of housing multiple worms (Figure S1a), each incubation arena contains Circular and Progeny chambers that are connected to Perimeter, Progeny, Flush, and Loading outlets (Figure 1b). Sieves at Progeny and Perimeter outlets prevent even the smallest L1 progeny from exiting the incubation arena. However, the sieves at the Flush outlet are big enough for the passage of L1 to L3 animals. Adult worms can only enter through Loading port that is connected to the bottom of a well and can be blocked by a pin (Figure 1c-d). Incubation units are connected in parallel to the chip ports ensuring uniform flow distribution (Figure 1e). Multiple PDMS layers are carefully aligned to create 200 incubation units (Figure S1b-d, Figure S2a-c). On-chip manifolds regulate flow direction through ports of each chip (Figure 1f). Moreover, a valve network controls fluid flow from reservoirs through the main feeding line into inlet chip manifold (Figure 1g). Manipulating these valves can generate flow patterns to carry out various tasks, including loading worms, flushing progeny out of chambers, and feeding or replacing incubation fluid (Figure 1h-j). In *Ce*Lab, setting Perimeter and Progeny ports as inlets, hatched L1-L3 progeny can be cleared from incubation arenas through the flush port in less than 10 minutes with nearly 100% success rate (Figure 1i, Video S1b-c).

Re-use of chips: In most microfluidic devices, a new chip must be made for each experiment (Table S1). By contrast, *Ce*Lab chips can be reused upon thorough cleaning. Running dilute chlorine bleach solution for a few minutes can break apart worm and bacterial residue from the chip (Figure 1g) and sterilizes the pipeline, eliminating the risk of contamination for long-term culture.

Loading: It is challenging to accurately deliver animals to intended locations in microfluidic chips[24] (Table S1); the hold-and-burst procedure can take 30 min with 80% yield[25], or 3 min with 65% yield[26]. Additionally, this highly size-dependent technique is stressful for worms as they are pushed through narrow openings. By contrast, the embedded well-plate feature of the *Ce*Lab chip offers a novel worm-loading technique; loading animals to *Ce*Lab chip is simple and size-independent with 100% yield (Figure 1h, Video S1a). Current worm-

loading techniques limit users to test one mutant per chip (Table S1). *Ce*Lab enables the user to load multiple mutants on a chip. After removing pins, wells can be filled in seconds. Deposited animals into wells can be pulled into incubation arenas nearly instantly by applying negative pressure (Figure 1h, Video S1a).

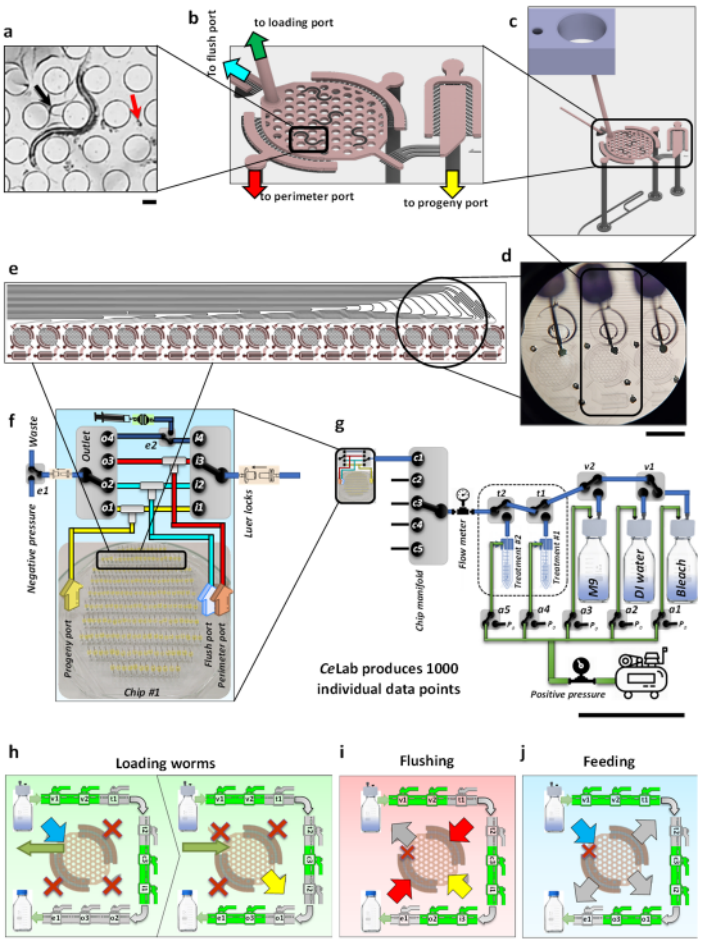


**Figure 1: *Ce*Lab, a miniaturized *C. elegans* Lab on a chip.** (a) Microposts, 200 µm in diameter with 300 µm spacing, allow animals crawl at a similar speed, wavelength, and amplitude as they do on an agar plate. Red and black arrows show fertilized egg and hatched progeny, respectively. Scale bar, 100 µm. (b) Each 3 mm diameter circular chamber can house multiple adult worms. Hatched progeny can be trapped in the "progeny chamber" to quantify daily progeny production. Unit number can be found next to the progeny chamber. (c) A 3D model of an incubation unit showing how different layers are connected. (d) Top-view image of wells and incubation area where pins block the loading ports. The smaller well, 1.5 mm in diameter, inside the 3 mm diameter main well, facilitates the worm loading process. Scale bar, 1 mm. (e) Identical hydraulic resistance across parallel channels connecting incubation arenas to flush, perimeter, and progeny ports ensures uniform flow distribution. (f) Flush, Perimeter, and Progeny ports can be either inlet, outlet, or closed by manipulation of on-chip inlet and outlet manifolds. Manipulating valves of inlet and outlet manifolds can generate various flow patterns inside the chip. (g) The main feeding line valve system of the *Ce*Lab control center directs flow from one reservoir into one chip at a time. Scale bar, 1 mm. (h-j) The schematics of the *Ce*Lab fluidic workflow demonstrates how generating different flow patterns within each incubation unit can accomplish various tasks such as (h) Loading worms, (i) Flushing progeny, and (j) Feeding.

**Table 1: *Ce*Lab accelerates experimental discovery.**

| Assay | # of worms | | Step 1 | Step 2 | Step 3 | Step 4 | Step 5 | Total |
|---|---|---|---|---|---|---|---|---|
| Lifespan | 1000 | Plates | Pouring plates | Seeding & labeling | Starting the experiment | Daily transferring | Scoring & Digitizing data | ~40 hr |
| | | ~750 (60mm) | 3 hr | 5 hr | 2 hr | 9 d × 2 hr | 12 d × 1 hr | |
| | | *CeLab* | Cleaning chip | Harvesting bacteria | loading animals | Daily flushing + Feeding | Scoring & digitizing data | ~6 hr |
| | | 1 chip | 10 min | 20 min | 1.5 hr | 16 d × 5 min | 10 d × 15 min | |
| Reproductive span | 200 | ~1600 (35mm) | 6 hr | 7 hr | 1 hr | 7 d × 1 hr | 9 d × 1 hr | ~30 hr |
| | | 1 chip | 10 min | 20 min | 1 hr | 10 d × 5 min | 8 d × 15 min | ~4 hr |

Bacterial feeding: Previous designs with continuous bacterial flow forms biofilm streamers that clog chips in a matter of days[27] (Table S1). Using less sticky *E. coli* strains (HT115) can delay the inevitable[28], but limits food options. Additionally, significant labor goes into preparing feeding solutions daily. In *Ce*Lab, incubation arenas are filled with feeding solutions once every day, eliminating the risk of clogging (Figure 1j, Video S1d). The bacterial solution is stored in-line and at 4°C for up to two weeks (Figure 1g, 1j). Only 1.5ml of feeding solution is required per chip per day, filling the chip in a matter of seconds (Table 1).

Scoring: Some current microfluidic technologies use tracking algorithms for computer-assisted scoring with pre-defined cut-off thresholds, and the quantifications must be reviewed for errors often making them as slow as manual scoring (Table S1). While suitable for scoring motility assays, they are subject to significant limitations, such as successfully identifying bagged or exploded worms. Although *Ce*Lab can be similarly imaged for automated size and motility measurements (Video S1e), it can also be scored manually on a dissecting scope for lifespan, reproductive span, and brood size, closely resembling the operation workflow of plate assays and can be easily adopted by biologists. To expedite manual scoring, we recently developed *Ce*Aid (***C. e**legans* **A**pplication for **i**nputting **d**ata)[29]. Implementing features such as voice command, swiping, and tap gestures, *Ce*Aid does not require the user to shift focus from the chip to the pen and paper throughout the assay (Table 1).

In addition to images, movies of individual wells can be made (Video S2), allowing automated analyses of movement, such as with CeleST[30], as we have used previously to identify age-related movement disorders[31–33].

### *Ce*Lab assays recapitulate plate assay results.

Lifespan assay: Devices that lack pillars often show short lifespans relative to plate assays, indicating that the worms are less healthy in the absence of "dirt"-like conditions[14]. To assess the ability of *Ce*Lab to mimic plate assays, we tested lifespans in the device. *Ce*Lab recapitulates previously-published lifespan results (Figure 2a, c). Moreover, the results are highly reproducible; following step-by-step operation guideline of

*Ce*Lab (Figure 1h-j), user-induced variability is eliminated, producing consistent results (Figure 2a, Figure S1e).

Each *Ce*Lab control center can hold up to 5 chips (Figure 1g, Figure S1c). Chips can be disconnected and incubated at different temperatures. As has been previously shown, worms kept at colder temps live longer (Figure 2b), while worms at higher temperatures live shorter; lifespans performed at these temperatures in *Ce*Lab also show such a temperature difference (Figure 2b).

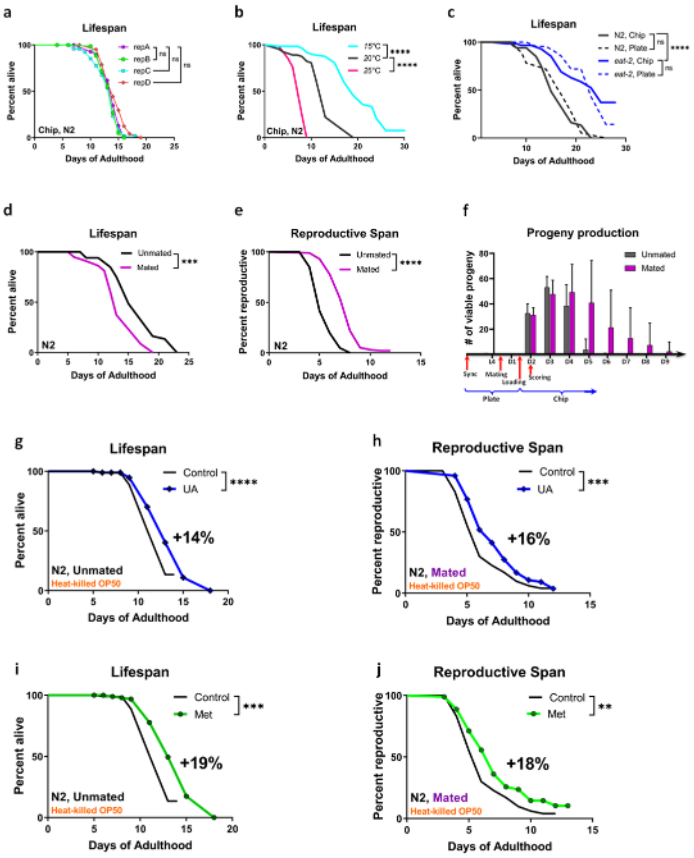


**Figure 2: *Ce*Lab reproduces previously-published plate assay results**. (a) An example of lifespan data from wild-type (N2) animals; *Ce*Lab's output data are highly reproducible, (repA, n=99; repB, n=80; repC, n=100; repD, n=66). (b) Wild-type (N2) lifespan at different temperatures. The *Ce*Lab chip replicates the normal lifespan of wild-type N2 worms at different temperatures (15°C, n=66; 20°C, n=66; 25°C, n=66). (c) As shown previously in plate assays, the dietary restriction mutant *eat-2* has an extended lifespan (N2 and *eat-2* on plates, n=80; N2 and *eat-2* in chip, n=66). (d) The *Ce*Lab chip also recapitulates shorter lifespan of mated animals (unmated, n=66; Mated, n=62) (Shi & Murphy, 2014) while (e) reproductive span (Unmated, n=101; Mated, n=100) and (f) progeny production (Unmated, n=51; Mated, n=51) are significantly increased. (g) 50 µM UA treatment in the *Ce*Lab chip replicates previously reported LS extension and (h) reproductive span extension (control, UA, n=100 in both g and h). (i) 50 µM Metformin treatment in the *Ce*Lab chip replicates previously reported LS extension. (j) Mated reproductive span extension is also extended with metformin treatment (control, Metformin, n=100 in both i and j). † indicates loss of hermaphrodites due to matricide. Kaplan-Meier survival tests. NS= not significant. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

We also tested the previously-studied dietary restriction longevity mutant *eat-2*, and found that *Ce*Lab recapitulates the extended lifespan previously observed on plates[34] (Figure 2c). These data also suggest that the worms are not calorically-restricted in the device; in such a case, we would have expected to see a lifespan extension of wild-type worms in the device, and no difference between wild-type worms and *eat-2* mutants in the device.

Mated assays: We previously found that while mating extends worms' reproductive span – because worms are able to use their remaining oocytes once self-sperm are exhausted – mating shortens lifespan[10,35]. Mated worms also produce more progeny later in life[36]. We are able to easily load adult worms and track individuals' progeny production and health; therefore, worms can be mated prior to loading in the device, and then we simultaneously measure lifespan, reproductive span, and progeny production (Figure 2d-f). It should be noted that not only was *Ce*Lab able to recapitulate the lifespan shortening, reproductive span extension, and progeny production increase upon mating previously shown in plate assays, but *Ce*Lab allows the measurement of these properties in the same individual animals.

Reproductive span: We can study reproductive aging by measuring the duration of the mother's ability to produce offspring daily. To measure reproduction manually, a single fourth larval stage (L4) hermaphrodite is placed on a petri dish with abundant food. Animals are then transferred to a fresh dish daily and are scored for reproductive span or the number of live progeny several days later. Manual reproductive span and brood size assays are highly laborious (Table 1). By contrast, *Ce*Lab is capable of testing 200 worms with any combination of mutants. Thrashing L1 to L3 progeny are easily visible within the incubation arena. During daily inspection, the user scans through the chambers (Video S1e) and records whether individual worms are reproductive by simply swiping up or down on *Ce*Aid. After logging data, hatched progeny are flushed out of the chip. With manual loading and scoring, RS assay using *Ce*Lab is about 7X faster than the manual assay (Table 1). Our *Ce*Lab results recapitulate earlier reproductive span results, including the increase in reproductive span after mating (Figure 2d).

Drug treatments: Many drugs and antioxidants have been shown to extend *C. elegans* lifespan[37,38]. Uptake of drugs by animals maintained on plates is an ongoing challenge[39] and plate assays need to take into account diffusion through the volume of the whole plate. It would be useful to be able to carry out high-throughput drug screens in *C. elegans* in small volumes. Each individual *Ce*Lab chip can hold different experimental fluids (i.e., drugs, RNAi, etc.) with any combination of mutants. *Ce*Lab chips enhance uptake of compounds in pharmacological assays, as animals are submerged in incubation solution. Additionally, only 1.5ml of feeding solution per chip per day is required, reducing the amount of drug required for treatment.

To test the drug screening capability of *Ce*Lab, we measured lifespans and reproductive spans of drug-treated animals. We tracked lifespan of wild-type animals treated with different "longevity" drugs (NMN, NAC, and Urolithin A) (Figure S1g-j; Figure 2g). The *Ce*Lab chip recapitulates

the extension of lifespan by NAC and Urolithin A that were previously published[40,41] (Figure S1g,h; Figure 2g). Additionally, Urolithin A extends mated reproductive span, as we had previously found[42] (Figure 2h), suggesting that the *Ce*Lab chip will be amenable for drug screening for both lifespan and reproductive spans.

**Metformin treatment increases lifespan and reproductive span.** We also tested metformin, the biguanide diabetes drug that has been reported to increase lifespan in a range of organisms, including *C. elegans*[43]; a recent study confirmed that metformin has lifespan and healthspan benefits for several (but not all) *Caenorhabditis* species[44]. A previous study had concluded that metformin's effects on *C. elegans* lifespan are mediated by perturbation of bacterial folate cycle metabolism, resulting in methionine restriction, rather than through metformin's effects on the worm itself [45]. However, those and prior assays were performed with very high (25-100 mM) levels of metformin in the plates. Reasoning that liquid treatment of metformin would be more effective than plate treatment, we reduced the dosage by a thousand-fold from previously published lifespan assays in *C. elegans*[43,45], and we used the same concentration (50 µM) that we had previously used in a drug screen to reduce Parkinson's-like phenotypes[31]. Note that in both Mor, et al. (2020) and here, we used heat-killed bacteria to avoid any effects caused by bacterial metabolism. We found that treatment with 50 µM metformin significantly increases both lifespan (+19%, p<0.001) and reproductive span (+18%, p<0.001) (Figure 2i, j). While metformin's positive effects on lifespan had been reported previously[43], *Ce*Lab allowed us to observe a simultaneous extension of reproductive span; these effects were observed at 1000-fold lower concentration of metformin than used for previous lifespan assays, but are similar to the positive effects we observed on suppressing Parkinson's-like behavioral and movement defects at this concentration of metformin[31].

***Ce*Lab reduces censored events, thus increasing assay information.** One of the problems that plagues *C. elegans* assays, particularly reproductive span assays, is the loss of animals due to matricide ("bagging"), which increases after mating and is exacerbated in many mutant conditions. When an animal bags, it must be censored from lifespan at that point, since it is not considered a natural death. Similarly, the animal should be censored just after bagging in a reproductive span, because the mother is still reproductive at that point but it is impossible to know how much longer she would remain so. Worms are also often censored from assays because they leave the bacterial spot and dry up on the walls of the plate or burrow into the agar ("lost"); some conditions and mutations increase the leaving rate of animals. The combination of leaving and bagging results in high censoring rates in many mated assays performed on plates. However, we find that censoring rates are much lower in the *Ce*Lab chip than on plates (Figure 3a). In addition to eliminating the possibility of leaving and thus reducing censoring due to missing animals, we noticed that worms in the *Ce*Lab chip were far less likely to bag (Figure 3b); this was true both for wild-type worms and for *daf-2* mutants, which generally bag at higher rates than wild-type worms in plate assays (Figure 3b); therefore, we were able to extend the time we can monitor the worms and thus better assess their full reproductive spans and make comparisons between one another. The extended reproductive span of *daf-2* animals is replicated in the *Ce*Lab assay, but with additional time points, as bagging rates are decreased (Figure 3c-d).

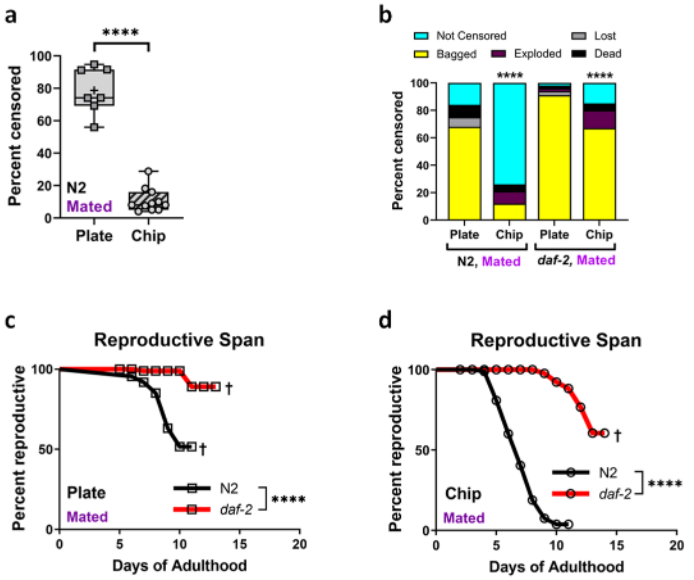


**Figure 3. *Ce*Lab reduces censoring rates in reproductive span assays.** (a) There are fewer censored events in the *Ce*Lab chip than in plate assays (plate, n=519 from 7 different experiments; Chip, n=852 from 11 different experiments). (b) The *Ce*Lab chip reduces different censoring events, but has the largest effect on the reduction of matricide (bagging, yellow) (Plate: N2, n=89; *daf-2*, n=106; Chip: N2, n=100; *daf-2*, n=196). (c, d) The extended reproductive span of *daf-2* worms is replicated in the *Ce*Lab chip. (Plate: N2, n=89; *daf-2*, n=106; Chip: N2, n=100; *daf-2*, n=196). Kaplan-Meier survival tests. Two-tailed t-tests. Chi-square test. ****p < 0.0001. Box plots show minimum, 25th percentile, median, 75th percentile, maximum.

**Correlational analyses of individual phenotypes.** *Ce*Lab can track hundreds of worms, generating both population analyses and data points for each measured individual phenotype (Figure 1g). Simultaneous measurement of visual biomarkers in individuals allows us to uncover new phenotypes and analyze possible correlations between characteristics in individuals, such as size and life history traits that are normally measured in populations (e.g., lifespan and reproductive span). We asked whether a worm's lifespan is correlated with a worm's body size, as previous studies have drawn opposite conclusions[46,47]. All body size measurements are done on day 7, which is at the end of the adult growth period[10,46,48]. Different body size parameters are correlated with lifespan in unique ways across the animals tested (Figure 4a, b): while there is no correlation between individual worm length and lifespan of wild type worms, individual *daf-2* worms show a slight positive correlation - that is, longer *daf-2* worms live longer - while longer germlineless *glp-1* animals live shorter (Figure 4a), despite the fact that there is overlap between these two genetic pathways[49]. Carrying out a similar analysis for width, we found that thinner wild-type and *glp-1*