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A microfluidic platform for lifelong high-resolution and high throughput imaging of subtle aging phenotypes in *C. elegans*

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Abstract

Aging produces a number of changes in neuronal structure and function throughout a variety of organisms. These aging-induced changes encompass a wide range of phenotypes, from loss of locomotion ability to defective production of synaptic vesicles. *C. elegans* is one of the primary systems to elucidate phenotypes associated with aging processes. Conventional aging studies in *C. elegans* are typically labor-intensive, low-throughput, and incorporate Fluorodeoxyuridine (FUDR) as a sterilization agent to keep the population age-synchronized throughout the assay. However, FUDR exposure induces lifespan extension, and can potentially mask the phenotypes associated with the natural aging process. In addition, studying cellular or subcellular structures requires anesthetics or adhesives to immobilize nematodes while acquiring high-resolution images. In this platform, we are able to maintain a population (~1000 worms) age-synchronized throughout its lifespan and perform a series of high-resolution microscopy in a drug free environment. The device is comprised of two main interconnected sections, one with the purpose of filtering progeny while keeping the parent population intact, and one for trapping nematodes in individual compartments for microscopy. Immobilization is carried out by decreasing the temperature of the device where nematodes are trapped by placing a heat sink on top of the chip. We were able to perform periodic high-resolution microscopy of fluorescently tagged synapses located at dorsal side of the nematode's tail throughout the worms' lifespan. To characterize the subtle phenotypes that emerge as nematodes age, computer vision was implemented to perform automated unbiased detection of synapses and quantitative analysis of aging-induced synaptic changes.

Graphical abstract

Conflicts of interest

There are no conflicts to declare

[†]Footnotes relating to the title and/or authors should appear here.

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Introduction

Human life expectancy has increased over the past century, and this pattern is expected to continue in next few decades¹. The main reason for this lifespan extension is improvement in healthcare. As a consequence of this life prolongation, the senior population, which is most susceptible to age-induced diseases including Alzheimer's, Parkinson's, and Huntington's, is growing². Thus, the fraction of people vulnerable to these illnesses is expected to increase in the near future, making aging studies crucial. However, studying this process directly in humans poses numerous difficulties, particularly the long timescale involved in the aging process. Model organisms, on the other hand, enable the study of aging in simpler systems with faster life cycle.

C. elegans, the first eukaryote to have its genome fully sequenced, is one of the premier model organisms to study aging^{3–5}. Its relatively short life-span (~20 days) enables rapid experimentation. These nematodes are self-fertilizers, which makes preparing a large isogenic population in 2–3 days, simple⁶. Their transparent bodies enable fluorescence microscopy of structures of interest with fluorescent tags^{7,8} in live, intact animals. *C. elegans* is amenable to easy genetic manipulation, especially with emerging novel gene editing techniques that have increased the efficiency and throughput of targeted insertion of markers and mutations⁹. Due to all these advantages, *C. elegans* has become a widely used model of aging, particularly after the discovery of mutations that dramatically extend lifespan¹⁰. Several aspects involved in aging and longevity have been studied in this model organism, such as DNA damage, mitochondrial dysfunction, autophagy impairment, and nutrient sensing dysregulation^{11,12}. Some efforts have also been devoted to explore neurodegeneration induced by aging, as *C. elegans* is also very important in neuroscience studies, mainly due to its fully mapped neuronal connections^{11,13–18}. Aging induces phenotypes reminiscent of functional deterioration in the nervous system: motility decline, long-term associative memory deterioration, and changes in neuronal morphology and synaptic patterning^{14,15,17–19}.

The nature of life-long aging studies in *C. elegans*, particularly those that require large sample numbers, makes them time consuming and labor-intensive. One of the key requirements of these studies is to maintain an age-synchronized population. To achieve this criterion, most aging assays commonly use 5-Fluorodeoxyuridine (FUDR) as a sterilizing agent, which prevents nematodes from laying viable progeny. However, studies have shown that FUDR interferes with longevity pathways and causes an artificial lifespan extension,

potentially affecting the aging phenotype of interest^{20,21}. As an alternative to FUDR, nematodes can be transferred from plate to plate on a daily basis to avoid mixing of progeny and parent population. This method suffers from being extensively time consuming and low throughput. In addition, aging studies that require high-resolution microscopy of cellular and subcellular features (such as neurons or synapses), also typically use anesthetics like Levamisole to achieve complete immobilization for imaging. However, Levamisole is a potent cholinergic agonist which paralyzes nematodes by binding to acetylcholine receptors, potentially inducing changes at the synaptic level^{22–24}.

To overcome these issues, various microfluidic platforms have been developed for imaging and aging studies²⁵. Xian et al. developed WormFarm, which enables long-term culture of an age-synchronized population without introducing FUDR²⁶. Hulme et al. designed a platform with 16 chambers connected to imaging channels where nematodes can be cultured and imaged individually²⁷. However, these platforms do not allow for high-resolution imaging of subcellular features. Various techniques for immobilization that avoid anesthetics on-chip include mechanical membrane deflection, cooling, CO₂ exposure, and temperature-driven gelation of a polymer. Integrating each of these methods with microfluidic devices has resulted in different levels of on-chip animal immobilization^{28–30}. A large number of microfluidic devices have also been developed to perform fluorescence microscopy³¹. Some examples include a device to perform high-throughput imaging and tracking of protein aggregation in a population^{32,33}, a multi-channel device to monitor cellular features in multiple L4 animals in parallel³⁴, platforms to image neuronal activity^{35,36}, and a platform to perform whole population phenotyping while tracking single individuals for high-content studies³⁷.

Despite the capabilities these systems provide, there is a need for a platform to track age-induced subtle phenotypes throughout the population's lifespan by conducting high-resolution high-throughput microscopy. In this study, we developed a platform to characterize aging of cellular and subcellular structures throughout a population's lifespan in a drug free environment (Figure 1.b). We focus on subtle changes in synaptic patterning in the dorsal axon of the DA9 neuron, a widely used model of synaptic patterning in *C. elegans*^{16,38}. We developed a platform that incorporates two interconnected sections: one responsible for evacuating progeny to keep the population age-synchronized, and a parallel array of channels for trapping and imaging individual worms (Figure 1.a)³⁴. In the first chamber, eggs and larvae are filtered out through hundreds of auxiliary channels surrounding the chamber while retaining the adult population, thus maintaining a population age-synchronized while avoiding the use of FUDR. These auxiliary channels are shallower than the rest of the chamber in order to prevent the adult worms' evacuation from the device while eggs are filtered out. Progeny removal is carried out once a day to ensure age-synchronization is maintained throughout the lifespan of the population. In the adjacent chambers, animals are loaded and immobilized for imaging. Three devices with different specifications were fabricated to accommodate the nematodes' size increase that occurs as they age (Figure 1.c), thus avoiding the possibility of worm injury in the system^{39,40}. High-resolution microscopy is performed once every three days by transferring nematodes to the imaging section. Nematodes are trapped individually in imaging compartments specially designed to restrict the motility of worms and favor a dorsal down orientation, thus

improving the image quality of DA9 pre-synaptic sites (Figure 1.d). Animals are then immobilized by cooling³⁰, placing a heat sink on the top of the device. By integrating this platform with a quantitative image analysis algorithm, we were able to quantitatively determine the phenotypic changes in fluorescent tagged presynaptic sites that occur as a result of aging. This novel platform enables high-resolution high-throughput imaging of small cellular and subcellular features as a population ages in a drug free environment. The high-throughput aspect of this platform enabled sampling a large set of animals from a large population, thus acquiring information from a subset with a phenotypic distribution representative of the entire population. The integration of quantitative analysis with high-throughput data acquisition enabled extracting rich information from images of sub-cellular features, leading to high-content deep characterization of subtle age-induced phenotypes.

Materials and methods

Device fabrication

To accommodate the change in size as worms age, three different designs with different specifications were created. Two separate molds were fabricated with different feature heights. To fabricate the first layer, SU-8 2025 was spun using a Laurell Spin Coater at 1750 rpm to obtain 40 μm tall features or at 1000 rpm for 80 μm features. To add the second 20 μm tall layer, the alignment marks of the first layer were developed before coating with SU-8 2010 spun at 1350 rpm for both designs. Following photo-lithography, soft-lithography was performed by pouring SYLGARD 184 silicone with 10:1 elastomer to crosslinker ratio on the silanized mold and allowed to cure overnight at 80°C^{41,42}.

Strain, media, culture

The strain used in these experiments (XA7810), kindly provided by Kang Shen, has fluorescent tags in the AWC neuron located in the head and in synaptic sites located at the dorsal cord of the DA9 neuron^{16,38}. The AWC neurons are tagged transcriptionally in red with mCherry (Podr-1::mCherry) and DA9 synapses are tagged translationally in green with GFP (Pitr-1 B::gfp::rab-3). To obtain an aged-synchronized population, eggs were extracted from gravid hermaphrodites using a bleach solution (1% NaOCl and 0.1 M NaOH)⁴³. The eggs were placed on seeded NGM plates upon extraction. Worms were cultured on plate for 2 days (until reaching young adulthood) at 25 °C and then transferred to liquid culture media for the rest of their life⁴⁴. The liquid culture media was made of *E. coli* OP50 suspended in S-medium. The bacteria were grown and incubated overnight in LB broth at 37°C. The suspension was centrifuged and LB was removed to acquire bacterial pellets. The pellets were washed thoroughly by S-medium, resuspended in S-medium, and stored at 4 °C. The culture concentration was diluted to 8×10^8 cells/ml ($\text{OD}_{600}=1$) by adding S-medium. A sterilized 0.01% solution of Triton X-100 (TX-100) in M9 was the media used for device operation. TX-100 was added to M9 as a surfactant to prevent adhesion of nematodes to tubing and the device.

Sterilization

For this device, an effort was made to avoid any drugs that are commonly used in conventional aging studies, such as antibiotics and antifungals used to prevent

contamination^{26,45}. To exclude drugs from the experiment, the entire process was performed in a sterile environment. To keep the process sterile, the media flown to the device was autoclaved prior to use. In addition, a 3-step sterilization of the tubing, connections, and the device was carried out. The first step was comprised of rinsing the inner parts of the tubing, connections, and the device by flowing 70% ethanol. In the second step, the tubing and device were packed unassembled in an autoclavable pouch⁴⁶. Autoclaving parts separated increased the likelihood of steam penetrating through to decontaminate. The pouch was autoclaved for 30 minutes followed by a 15-minute drying cycle. The pouch was then transferred to Thermo Scientific 1300 Series A2 biosafety cabinet and was UV irradiated for 2 hours⁴⁷. To minimize the chance of contaminating the setup, the pouches were opened prior to the experiments in a biosafety cabinet, and assembly was carried out under the hood. The entire platform was always kept in the biosafety cabinet and was only transferred for experiments. The air flown into the conical tubes was filtered by 0.22 μm syringe filters as an extra precaution.

Microscopy

Daily progeny evacuation was performed in brightfield using a Leica DMI8 inverted scope with a 5x objective. Prior to high-resolution microscopy sessions, worm loading was performed and inspected under a 5x objective to assure proper loading. High-resolution microscopy was carried out using 40x/1.30 oil objective. Confocal fluorescence microscopy was performed with an LDI Laser Launch coupled with a CrestOptics X-Light V2 confocal imager and an Orca-Flash 4.0 digital CMOS camera. The exposure time and laser intensity were kept constant through all experiments at 60 ms and 40% respectively. For each individual nematode, images with 31 slices (Z stacks with 1 μm spacing) were acquired with Micro Manager and the maximum projection of these slices was further processed with MATLAB.

Results and discussion

To characterize the aging behavior of DA9 dorsal cord synapses at different time-points throughout a population's lifespan, periodic high-resolution microscopy of an age-synchronized population was performed in the platform. A population of ~1000 worms was cultured throughout its lifespan while progeny were filtered out of the device to keep the population age-synchronized. Once every three days, nematodes were transferred to the imaging section and high-resolution fluorescence microscopy was performed. Images were segmented, quantified, and analyzed to extract the relevant information incorporated in each image.

On-chip high-throughput age-synchronization for drug-free lifelong culture

C. elegans' lifespan on-chip at 25 °C is ~16 days and they are fertile for 5 days, from day 2 (egg laying adult) to day 7^{27,48}. It takes 2 days for an egg to reach the egg-laying adulthood phase. Morphological similarities among day 3 and day 5 animals make distinguishing them almost impossible. Thus, aging studies require all individuals within a population to be the same age (i.e., age-synchronized) throughout the assay. To achieve this goal throughout the lifespan of the population, worms were grown in liquid culture flasks for 24 hours at 25 °C

and transferred daily to the device for progeny filtration. The main chamber is surrounded by hundreds of auxiliary channels (Figure 2.a) which allow progeny removal, thus keeping the population age-synchronized in the absence of FUdR. The auxiliary channels were precisely designed to address the complexity of mechanically filtering progeny, as the width difference between eggs and adult animals is only $\sim 20\ \mu\text{m}$. In addition, the nematodes' motility poses an additional difficulty, since they can squeeze through small openings. An ideal sieve should be wide enough to let the progeny pass while also being tapered and long to prevent evacuation of adult animals. Thus, the auxiliary channels surrounding the chamber were designed to be tapered, long, and shallower than the main chamber. This configuration prevents animals from squeezing through while allowing egg removal.

As previously mentioned, we developed three devices with different dimensions (Devices 1–3) as the size of the animal continues to increase throughout adulthood. As shown in Table 1, several widths and lengths were tested, and the optimal design was selected. The height of the chamber for animals at the earlier stages (Device 1) was fabricated to be $60\ \mu\text{m}$ in height, compared to $100\ \mu\text{m}$ for later stage animals (Devices 2–3). Similarly, the auxiliary channels were 40 and $80\ \mu\text{m}$ in height, respectively. The auxiliary channel entrance width was $45\ \mu\text{m}$ and tapered down to $35\ \mu\text{m}$ at the exit (Figure 2.b) in both devices. The channel length was $600\ \mu\text{m}$ to minimize the probability of adult nematodes leaving the chamber and avoiding a significant increase in pressure drop. The first step in the progeny filtration process involved transferring the population from the liquid culture flask to centrifuge tubes, and washing with M9 buffer 2–3 times. This step reduced the amount of progeny to be removed by 40–50%, thus simplifying egg evacuation. The nematodes were then introduced to the main chamber where bidirectional flow from the two ends of the device (Figure 3. a, b, c) flushed the progeny through the auxiliary channels to the outlet connected to a waste container. The filtration process was continued until all eggs and larvae were completely removed from the sample, as evaluated by visual inspection. Worms were then either transferred to the imaging section or back to the culture flasks depending on the upcoming task.

Loading and trapping nematodes for high-resolution imaging

High-resolution microscopy of subcellular structures such as synaptic puncta, requires minimal movement of the sample. Worm locomotion complicates the process of high-resolution imaging, since small displacement results in poor quality, unreliable images. To decrease their motility, animals were individually directed to separate compartments that restrict their movement. The size of the nematodes plays a crucial role in designing these channels, since loading efficiency (number of worms loaded/ number of channels) decreases drastically if the channels are either larger or smaller than the worms. As previously mentioned (Figure 2.b), three different designs were fabricated to address the size increase of nematodes as they age. Microscopy performed at days 3 and 6 were performed in Device 1, while Devices 2 and 3 were used at days 9 and 12 respectively.

Animal orientation during imaging is crucial due to the anatomical location of the structure of interest. Nematodes are usually on their lateral side, particularly while crawling on solid media. However, as shown in Figure 1.d, the optimum orientation to image synapses located at the dorsal nerve cord is with the dorsal side close to the glass slide. In the case of wrong

orientation (ventral side down), the light must travel through the tissue to reach the synapses at the dorsal side, making imaging unfeasible. To address this issue, we incorporated a height restriction (Figure 2.b) at the beginning of each imaging channel which increases the probability of positioning the animals at the desired orientation⁴⁹, and helps push animals towards the coverslip.

Loading occurs as a result of a pressure gradient across the two ends of the imaging channels. Continuous flow through the imaging section and each channel created the hydrodynamic force needed to maintain the animals in the compartments as microscopy took place (Figure 3a, d, e). Different designs and iterations were tested to optimize the loading parameters which include: loading efficiency, orientation, and escape prevention. The platform with optimized specifications achieved a consistent ~90–95% loading efficiency (Table 2). In addition, the height restriction at the beginning of each channel and the width reduction (8 μm) at the end of the imaging compartments, led to a better orientation and fewer worms leaving the imaging compartments during microscopy compared to channels with wider ends and no height restriction.

Temperature induced immobilization

As previously mentioned, the tapered imaging channels restricted the motility of the nematodes to a certain extent. However, to perform high-resolution microscopy, complete immobilization is required. *C. elegans* can be immobilized by reducing the surrounding temperature. To decrease the temperature in the imaging channels, a dry ice pellet was placed on the top of the platform as a heat sink after loading and trapping the nematodes. To ensure that immobilization has taken place as result of temperature reduction and not carbon dioxide diffusion, the PDMS surface was covered with a glass coverslip, thus preventing carbon dioxide permeation (Supplemental Figure 1. a). The temperature in the imaging section can be manipulated by the flowrate of room-temperature media introduced to the channels, since it counteracts the effect of dry ice. Higher flowrates lead to shorter fluid residence time, reducing the amount of heat removed from a certain control volume. On the other hand, media with a lower flowrate has more time to be in contact with the cooled PDMS surfaces, allowing more heat to be removed and lowering the temperature. As shown in Supplemental Figure 1.b, the flow rate was initially high, and reduced step-wise until immobilization occurred. Higher flowrates were used in the initial steps to avoid freezing. After each flow reduction step, the nematodes' motility was assessed, and flow was reduced further if necessary. The flowrate was decreased until full immobilization of nematodes was observed. In this way, we achieved simultaneous drug free immobilization of up to 72 nematodes.

Evacuation

After immobilization and microscopy, nematodes can be retrieved and evacuated from the imaging channels as well as the platform. Dry ice was removed from the platform after imaging was completed to raise the temperature. Nematodes were kept in the imaging channels for a few minutes until they regained motility. To evacuate worms, a backward flow was introduced (Figure 3.a) to direct the nematodes out of the imaging channels to the main

chamber and then out of the device to a collection vial. The population was then resuspended in the liquid culture flask and incubated at 25 °C.

Analysis of drug-free synaptic aging

To track how aging alters synaptic sites, 4 microscopy sessions were conducted at days 3, 6, 9, and 12. The strain used in this experiment (XA7810), has a transgene that expresses RAB-3 tagged with GFP. The *rab-3* gene encodes a synaptic vesicle membrane protein that localizes to presynaptic sites. Hence, this fusion protein allows for characterization of the morphology of presynaptic sites, and in this case, to assess the effects of aging on presynaptic patterning. Some of these age-induced phenotypes can be identified by visual inspection. However, a large amount of changes exhibited by such small (~1 µm) structures are expected to be subtle, making in-depth quantitative analysis of multiple descriptive metrics necessary. Computer vision made identifying and tracking subtle age-induced changes that the synaptic domain undergoes possible by detecting synapses, extracting quantitative metrics, and comparing these measurements at various time points. The relatively higher sample population accompanied by the unbiased analysis algorithm used in this study resulted in high accuracy and confidence in recognizing subtle phenotypes that arise as the population ages. Different aspects of synaptic aging were studied by extracting over 60 metrics from the images (Supplemental information). These metrics mainly unveil how the size, intensity, density, and distance of synapses change as nematodes age. Here, both qualitative and quantitative aspect of presynaptic aging have been studied.

Qualitative analysis of synaptic changes

The images acquired of the synaptic domain revealed several changes in synapse morphology as nematodes age. In young individuals, the DA9 synapses are located at a specific segment of the axon at the dorsal side of the tail. Li et al. have previously revealed the migration of presynaptic sites (puncta) toward the dorsal anterior synaptic region, the commissure, and even to the dendrite in aged individuals¹⁶. The images taken from 12-day old nematodes in the device (Figure 4.a) also showed this phenotype. Sequential analysis of synaptic aging revealed an increase in puncta irregularities, such as mis-localization of puncta and less uniform puncta orientation. As highlighted in Figure 4.b (red boxes), small unorganized synaptic sites tend to form in the vicinity of other synapses, which is not observed earlier in the nematode's life-span. In addition, the localization of synapses in the axon also underwent changes. In some cases, less uniform distribution and uneven orientation of synaptic vesicles (Figure 4.b, cyan boxes) was observed later in life, which suggests that the mechanism responsible for maintaining synaptic puncta location and morphology is impaired. In addition to arbitrary distribution of the vesicles along the axon, a segment of the axon itself tends to bend and form a curve rather than a line in a fraction of the aged population (Figure 4.b, green line). None of the images acquired at earlier stages showed such a phenotype. These irregular axon deflections started in day 6 in a subtler form and progressively aggravated.

Image processing and quantitative analysis of the data

Neuronal aging can result in drastic morphological changes in neurons, as well as drastic and subtle synaptic patterning and morphology changes. The more evident phenotypes can

be identified by qualitatively assessing the data acquired, as previously shown. However, qualitative image analysis and assessment is subject to binarized and biased decision-making, which is inadequate for subtler phenotypes. Most of the age-induced changes in synapses studied in this paper are expected to be very subtle and thus difficult to identify by visual and qualitative inspection. In addition, phenotypes such as synaptic distribution, localization, and size need unbiased quantitative measurement in order to be compared at different time points. To address these issues, an automated algorithm was exploited to detect synapses and extract descriptive metrics, such as average puncta intensity, puncta size, interpunctal distance, etc.

Detection of synapses was carried out by a machine learning model (Support Vector Machine) developed by San Miguel et al⁴⁹. This algorithm is a trained model to distinguish synaptic patterns from auto-fluorescence and background noise (Supplemental Figure 2). In addition to the raw image, additional input information required for the model to distinguish the synapses includes 25 different filtered images (such as a low pass Gaussian, Laplacian, average, etc.). These filters are used to enhance different aspects of the image, enabling the model to determine whether a pixel is part of a synapse or not. The segmented images were then used to evaluate the metrics discussed earlier. More than 60 metrics were calculated that describe how synaptic size, intensity, density etc., change over time.

Average puncta size, mean of average puncta intensity, and puncta density

The average puncta size is an indirect measurement of the synaptic sites available at presynaptic domain and is measured by the number of pixels it occupies. The average puncta size of the population imaged exhibited an overall increase through time (Figure 5.a, b). The puncta enlargement started between days 6 to 9 and was stabilized with minor change from day 9 to day 12. There is a significant increase of mean size between the days 3 and 12 ($P < 0.001$), suggesting an accumulation of synaptic vesicles. In parallel with puncta size growth, puncta fluorescence intensity changed in a similar fashion as puncta size, showing an evident increase between days 6 and 9 (Figure 5.a, b). The increase in intensity of synaptic puncta can be an indication of more vesicles available at presynaptic sites, which hypothetically could lead to more neurotransmitter available. However, studies have shown that both the locomotion rate and synaptic transmission declines in aged worms due to a reduction in kinesin-driven vesicle trafficking (reduced unc-104 expression)^{14,16}. This is counter-intuitive with a potential increased concentration of the vesicles at the domain. The accumulation of vesicles can be as result of a reduction in the rate of vesicle recycling to the cell body as nematodes age, which would lead to vesicle build up in presynaptic compartments. In this scenario, the reduction in locomotion could be caused by fewer neurotransmitter-filled vesicles delivered to the presynaptic sites, or a decline in postsynaptic receptor function. However, it is also possible that the observed intensity increase is a result of transgene over-expression. Further tests with endogenously labelled strains will enable testing this hypothesis.

Our data suggests that the synaptic density (number of puncta / total distance of synaptic domain) is reduced in the aged population (Figure 5.a, b). This data recapitulates previous results where an aging-induced decrease in synaptic density was concluded to be a result of

lower unc-104 neuronal expression¹⁶. The lower density could also be caused by the animals' size increase, assuming this results in an extension of the axon greater than the addition of synaptic sites.

Overall synaptic degradation

The algorithm used in this study enabled us to quantify and compare over 60 metrics (listed in supplemental information) during the population's aging process. These metrics describe changes in intensity, size, density, and distance of synaptic sites. To analyze and track the changes throughout the lifespan, a multiple comparison (Tukey-Kramer) one-way ANOVA of each measurement was conducted to extract p-values associated with each day compared to day 3 (Figure 6.a). As discussed earlier, average puncta size (metric 2) and mean of average puncta intensity (metric 20) undergo a significant change starting from day 9 of adulthood. Puncta density (metric 33) and mean of interpunctal distance (metric 30) on the other hand, remain intact until day 9 and the significant changes start to occur from day 12. Overall, the probability values for the first 3 days of adulthood (days 3–6) imply no major change in synapse morphology. Only 2 metrics showed a significant change ($p\text{-value} < 0.05$) suggesting that the overall synaptic structure in dorsal nerve cord is maintained during that period. The most drastic degeneration occurred between days 6–9, where the probability values of more metrics start to decrease. The shift of probability values to lower values is indicative of a higher chance of exhibiting a structurally different synaptic domain later in life. The Z-score, which is a standardized measurement for mean difference, at day 9 versus day 3 also confirms a major change in average values in some metrics (Figure 6.b). Consistent with prior results, a few more significant changes occurred from day 9 to 12, implying that more age-induced phenotypes continued to appear, although at a smaller rate. To identify the metrics with drastic changes, we counted the number of metrics with $p > 0.05$ (NS), $0.01 < p < 0.05$ (*), $0.001 < p < 0.01$ (**), and $p < 0.001$ (***) at each time point. As anticipated, day 12 has the most metrics with significant changes. In addition, the number of metrics with drastic changes ($p < 0.001$) is higher at day 12 compared to day 9, which implies even the phenotypes formed by day 9 become bolder (**Figure 7**). For instance, the maximum integrated intensity (metric 28) had already exhibited a significant change at day 9 with a P value of ~ 0.025 . The difference became even more drastic at day 12 where the p-value reached $7E-07$.

Conclusion

Performing high-throughput high-resolution aging studies on *C. elegans* with a large sample size poses various technical difficulties. In this study, we designed and fabricated a microfluidic platform capable of acquiring high-resolution images of subcellular features in a drug free environment. We were able to accomplish 3 main goals with this platform. First, we kept a large population (~ 1000 worms) age-synchronized throughout their whole lifespan without the need to expose the population to FUDR, which has been known to result in artificial lifespan extension^{20,21}. In addition, the use of antibiotics to prevent the growth of any external bacteria or fungi was replaced with a 3-step sterilizations technique. Second, $\sim 60\text{--}70$ nematodes were able to be trapped and immobilized simultaneously per run allowing for the use of microscopy to acquire images of synaptic domain located at dorsal

nerve cord. Immobilization was accomplished by establishing a temperature gradient instead of anesthetic drugs such as Levamisole, which may have a side effect at the synaptic levels^{22,23}. Third, subtle aging-induced phenotypes in the synaptic domain were quantitatively characterized using computer-assisted unbiased image processing tools. This platform will thus enable studying age-induced phenotypes at cellular and subcellular levels in *C. elegans* in a quantitative, drug-free, and high-throughput manner. The throughput of progeny evacuation, trapping, and immobilization can be even improved by automating device scanning for imaging, and by parallelizing multiple devices.

In this work, we focused on high-content characterization of DA9 synapses. This platform will enable future studies to deepen our understanding of neuronal aging. In particular, the use of this device can be extended to studying the cause of impaired locomotion and reduced synaptic signaling thoroughly by tagging different proteins involved in synaptic transmission, such as proteins in the pre and post synaptic region. For instance, we expect that trans-synaptic labeling⁸ will enable us to determine whether reduced locomotion is a result of loss of neuronal connectivity, which could also explain the increase in synaptic sites we observe. Nonetheless, this platform has wide applicability, as it enables aging studies at cellular and subcellular resolution in a wide variety of cell types. Some examples include age-dependent neuronal degeneration, protein aggregation, muscle deterioration, fat accumulation, and germline health. The results acquired using this platform can be coupled with behavioral analysis to establish a correlation between cellular and subcellular phenotypes with functional outputs, such as locomotion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Microfluidic platform for high-resolution high-throughput imaging of subcellular aging phenotypes. a) Photograph of microfluidic platform. b) Entire experimental imaging system. Media flow through the device was controlled by a custom built pneumatic pressure box that regulates pressure in the stock media bottles. Confocal fluorescence microscopy was carried out on an inverted microscope equipped with a motorized stage. c) Nematodes increase in both width and length throughout the aging process. d) Nematodes are usually on their lateral side, which is not a desired orientation for microscopy of synaptic sites located at the dorsal axon. This device favors a desired dorsal-down orientation.

Figure 2. Overview of the platform design specifications. a) The device is made of two main sections: a main chamber and an imaging section. The main chamber is comprised of a chamber surrounded with hundreds of auxiliary channels fabricated to evacuate the progeny and keep the population age-synchronized. 72 traps are designed in the imaging section where nematodes are individually trapped for imaging. b) Detailed specifications of different designs. The auxiliary channels are designed to be shallower than the rest of device and tapered to prevent adult population from exiting the chamber. The imaging channels are also designed to be tapered, lowering the motility of worms. A height restriction at the beginning of channels favored dorsal down orientation. The table lists the device used for each specific period of a population’s lifespan. Scale bar is 5 mm.

Figure 3.

Operational loop of the platform. a) There are 2 operational regimes carried out, depending on the desired task. Daily progeny evacuation was carried out once a day while nematodes were reproductively active. Nematodes were flown into the main chamber, eggs and larvae were evacuated, and the population was evacuated from the platform and transferred to the culture media. Complete imaging cycles were performed on day 3, 6, 9, and 12 when periodic microscopy was scheduled. b) Population in the main chamber during progeny evacuation. Larvae and eggs are evacuated from platform. c) Progeny evacuation (red arrow shows progeny getting evacuated). d) Nematodes were transferred to the imaging section and trapped in each imaging compartment. e) Worms loaded in each trap. Scale bars are 2, 1, 2, and 0.5 mm, respectively.

Figure 4.

High-resolution microscopy of synapses located at the DA9 axon. a) Puncta formation along dorsal asynaptic region, commissure, and dendrites is exhibited in aged individuals (Lateral view, yellow dashed boxes). Migration of synaptic sites toward the cell body and dendrite initiated at day 9 and aggravated by day 12. b) Major morphological changes occur throughout the aging process (dorsal view). Small synaptic sites tend to form in the vicinity of larger sites (Red dashed boxes). Uneven and irregular distribution of sites along axonal cord (cyan dashed box). The axonal cord seems to bend and form a curve in aged nematodes (green line). Scale bar is 50 μ m.

Figure 5.

Quantitative analysis of acquired images. a) Characterization of synaptic aging throughout the population's lifespan. Intensity, size, and density of synapses were chosen as representative metrics. b) Binning of the results acquired for average intensity, size, and density. The Gaussian distribution was calculated and plotted for each data set by Origin Lab. $p > .05$ (NS), $0.01 < p < 0.05$ (*), $0.001 < p < 0.01$ (**), and $p < 0.001$ (***). (N3=35, N6=25, N9=28, N12=41). Error bars are SEM. All p-values were calculated by multiple comparison one-way ANOVA using MATLAB.

Figure 6.

Deep phenotyping of subtle changes of DA9 dorsal axon synaptic sites. a) P-values calculated from one-way ANOVA comparison to day 3. p-values were corrected using multiple comparison method (Tukey-Kramer). b) Heatmap of Z-scores calculated at each time point, using day 3 as the baseline. (Purple boxes represent metrics discussed in Figure 5) (Brown box represents the metric for mean of interpunctal distance) (Black box represents the metric for maximum integrated intensity)

Figure 7. Characterization of synaptic aging. Binning of p-values calculated at each time point. $p > .05$ (NS), $0.01 < p < 0.05$ (*), $0.001 < p < 0.01$ (**), and $p < 0.001$ (***).

Table 1

Device designs tested to optimize progeny evacuation. (*) Successful task, (–) Failed tasks.

Width (Entrance /Exit) (μm)	Length (μm)	Progeny evacuation	Adult escape prevention	Pressure drop
20/20	150	-	-	Low
20/20	250	-	-	Low
40/25	280	*	-	Low
45/27	400	*	-	Medium
45/30	400	*	-	Medium
45/30	600	*	*	Medium
45/30	800	*	*	High

Table 2.

Loading efficiency of imaging section in different runs.

	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
Loading efficiency (%)	90	86	96	97	95