Tracking mitochondrial density and positioning along a growing neuronal process in individual *C. elegans* neuron using a long-term growth and imaging microfluidic device

https://doi.org/10.1523/ENEURO.0360-20.2021

Cite as: eNeuro 2021; 10.1523/ENEURO.0360-20.2021

Received: 16 August 2020
Revised: 18 April 2021
Accepted: 6 May 2021

This Early Release article has been peer-reviewed and accepted, but has not been through the composition and copyediting processes. The final version may differ slightly in style or formatting and will contain links to any extended data.

**Alerts:** Sign up at [www.eneuro.org/alerts](http://www.eneuro.org/alerts) to receive customized email alerts when the fully formatted version of this article is published.

Copyright © 2021 Mondal et al.
This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license, which permits unrestricted use, distribution and reproduction in any medium provided that the original work is properly attributed.
Title: Tracking mitochondrial density and positioning along a growing neuronal process in individual *C. elegans* neuron using a long-term growth and imaging microfluidic device

Abbreviated title: Mitochondria tracking using a microfluidic device

Authors and affiliations: Sudip Mondal,1,2,* Jyoti Dubey,1,3,4 Anjali Awasthi,1,5 Guruprasad Reddy Sure,1,6 Amruta Vasudevan3, and Sandhya P. Koushika3,*

1National Centre for Biological Sciences, TIFR, Bangalore, Karnataka, 560065, India. 2Department of Mechanical Engineering, The University of Texas at Austin, Texas, 78712, USA 3Department of Biological Sciences, TIFR, Mumbai, 400005, India. 4InSTEM, Bangalore, Karnataka, 560065, India and Manipal Academy of Higher Education, Manipal, Karnataka, 576104, India. 5Department of Biological Sciences, BITS Pilani, Rajasthan, 333031, India. 6Sastra University, Tamil Nadu, 613401, India.

Author Contributions:

SM and SPK designed the experiments. SM designed the device, SM and JD fabricated the devices. GPR and AA performed initial mitochondria imaging on agar pads using an anesthetic. SM characterized the device for long-term imaging and carried out the on-chip growth experiments. SM and JD performed long-term mitochondria imaging and analyzed the imaging data. JD performed and analyzed the mitochondria photo-bleaching experiments. AV performed time-lapse imaging of animals immobilized with levamisole in the device and JD analyzed the data. SM performed the simulations, probability tests, and statistical analysis. SM and SPK wrote the paper.
*Correspondence: Sudip Mondal (sudipmondal1979@gmail.com) and Sandhya P. Koushika
(spkoushika@tifr.res.in)

Lead contact: Sandhya P. Koushika (spkoushika@tifr.res.in)

Acknowledgments

SPK made jsIs609 in Mike Nonet’s Laboratory. We acknowledge Anyaa Mittal and Arpan Agnihotri for their help with microfluidic device fabrication and growing C. elegans inside microfluidic devices. We are grateful to Gautam Menon for useful discussion on mitochondria transport imaging and image analysis. We would like to thank Sangeetha Iyer (Perlara Labs) and Souvik Modi for suggestions on our manuscript. We are thankful to the reviewers for their thoughtful comments. We thank the CIFF imaging facility, NCBS for use of the confocal microscopes supported by the DST – Centre for Nanotechnology (No. SR/55/NM-36-2005).

Conflict of Interest: Authors report a conflict of interest. S.M. and S.P.K. are authors of a pending patent on the microfluidic growth and imaging device (Patent application 640/CHE/2011).

Funding sources: We thank research funding from DBT (SPK), DST (SM), DBT (SM), CSIR-UGC (JD), spinning disc supported by DAE-PRISM 12-R&D-IMS-5.02.0202 (SPK and Gautam Menon), and HHMI-IECS grant number 55007425 (SPK).
Abstract

The long cellular architecture of neurons requires regulation in part through transport and anchoring events to distribute intracellular organelles. During development, cellular and subcellular events such as organelle additions and their recruitment at specific sites on the growing axons occur over different time scales and often show inter-animal variability thus making it difficult to identify specific phenomena in population averages. To measure the variability in sub-cellular events such as organelle positions, we developed a microfluidic device to feed and immobilize *C. elegans* for high-resolution imaging over several days. The microfluidic device enabled long-term imaging of individual animals and allowed us to investigate organelle density using mitochondria as a testbed in a growing neuronal process *in vivo*. Sub-cellular imaging of an individual neuron in multiple animals, over 36 hours in our microfluidic device, shows the addition of new mitochondria along the neuronal process and an increase in the accumulation of synaptic vesicles at synapses. Long-term imaging of individual *C. elegans* touch receptor neurons shows that the addition of new mitochondria takes place along the entire neuronal process length at a rate of ~0.6 mitochondria per hour. The threshold for the addition of a new mitochondrion occurs when the average separation between the two pre-existing mitochondria exceeds 24 μm. Our assay provides a new opportunity to move beyond simple observations obtained from *in vitro* assays to allow the discovery of genes that regulate positioning of mitochondria in neurons.

Keywords

*C. elegans*, touch receptor neurons, mitochondria, intermitochondrial distances, development, microfluidic device, long-term imaging.
Significance Statement

Axonal transport of mitochondria is required for the normal function and health of a developing animal with continuously growing axonal processes. Existing technologies are unable to monitor the addition of a new mitochondrion in a growing axon \textit{in vivo}, as it requires continuous or intermittent tracking of the same individual neuron over several hours to days. We have developed a microfluidic device that enables long-term high-resolution imaging of individual \textit{C. elegans} in an anesthetic-free setting. Using this device, we observe that the addition of a new mitochondrion can occur anywhere along the entire neuronal process, likely mediated by actively transported mitochondria, and at docking sites that occur with high probability when the separation between adjacent mitochondria crosses 24 μm threshold.
Development and maturation of neurons occur through the processes of cell birth, cell differentiation, cell migration, neuronal process outgrowth, dendritic arborization, synaptic growth, and organelle transport (Smith et al., 2010, Hobert, 2010, Varier and Kaiser, 2011, Smith and Gallo, 2017). Complex cellular architectures present in polarized cells such as neurons need the transport of organelles to support many energy-dependent processes such as neurotransmission (Sheng and Cai, 2012). One such organelle is mitochondria that are essential for neuronal function and thought to contribute to various disease pathologies (Liu et al., 2012, Millecamps and Julien, 2013). Only ~20% of a large number of mitochondria in a neuronal process move and their halting depend on molecular motors, calcium, and other signaling molecules (Chen and Sheng, 2013, Kang et al., 2008, Sun et al., 2013, Schwarz, 2013, Misgeld and Schwarz, 2017). Mitochondrial trafficking continues throughout development when neurons undergo elongation over several fold and offers a unique opportunity to demonstrate the utility of long-term imaging in individual neurons. Time-lapse imaging of rapidly transported mitochondria in neuronal cultures over a few hours has identified that moving mitochondria preferentially dock between pairs of pre-existing docked mitochondria (Miller and Sheetz, 2004) and thus help maintain a uniform mitochondrial distribution along axons (Miller and Sheetz, 2004, Misko et al., 2012). Thus far, many invertebrate axons have not typically been reported to have general anatomical features such as nodes of Ranvier enriched in mitochondria as observed in vertebrate neurons (Ohno et al., 2011, Chiu, 2011). Multiple neurons, both in vivo and in non-myelinated neurons in culture, show that the density of mitochondria is invariant (Misgeld and Schwarz, 2017, Wang and Schwarz, 2009a, Morsci et al.,
The majority of mitochondria in *C. elegans* touch receptor neurons (TRNs) have been reported to be present at actin-rich regions (Sood et al., 2018) and the density of mitochondria in adult animals remains largely invariant (Sure et al., 2018, Morsci et al., 2016). Despite averages being tightly regulated, inter-animal variability is shown to exist even in nearly invariant cell lineages (Keil et al., 2016). Developing and aging neurons show variability in mitochondrial density and axonal length in a clonal genetic population (O'Toole et al., 2008), show heterogeneity of mitochondrial dynamics within individual cells (Sison et al., 2017), and show differential mitochondrial dynamics and distribution within different neuron segments (Obashi and Okabe, 2013). To investigate where mitochondria are added *in vivo*, one needs to analyze each mitochondrion along the entire neuronal process length, which can elongate significantly i.e. >5 fold over ~3 days in the same *C. elegans* animal.

Developmental events can be easily tracked in *C. elegans* using a microfluidic platform. Several microfluidic platforms have been developed for *C. elegans* to carry out high-resolution imaging (Chalasani et al., 2007, Caceres Ide et al., 2012, Mondal et al., 2012, Mondal et al., 2011), high-throughput screening (Chung et al., 2008, Mondal et al., 2016, Mondal et al., 2018), long-term imaging (Hulme et al., 2010, Krajniak and Lu, 2010, Keil et al., 2016, Xian et al., 2013, Atakan et al., 2019), and neuronal axotomy (Guo et al., 2008, Samara et al., 2010). Microfluidic device immobilization avoids alterations in *C. elegans* physiological processes caused by anesthetic immobilization (Guo et al., 2008, Mondal et al., 2012, Mondal et al., 2011, Mondal and Koushika, 2014). Anesthetic-free immobilization can prevent mechanical stress induced by chemical immobilization methods that change muscle tone and body stiffness, leading to artificial elongation or contraction of the overall body length (Petzold et al., 2011).
can also be cultured by feeding them with bacterial cultures inside microfluidic devices (Krajniak and Lu, 2010, Xian et al., 2013, Atakan et al., 2019, Krajniak et al., 2013, Lee et al., 2014, Gokce et al., 2017). Microfluidic devices allow time-lapse neuronal imaging in well-fed animals for up to 24 hours (Lee et al., 2014). In aging studies, long-term microfluidic devices have been used to grow *C. elegans* and acquire low-resolution images to quantify brood size and monitor locomotion (Xian et al., 2013, Atakan et al., 2019, Rahman et al., 2018). Recently, neuronal branching dynamics and lineage patterns were monitored using an imaging platform from an animal growing over 3 days (Keil et al., 2016). As yet, there is no study tracking sub-cellular events long-term such as monitoring organelle distributions or accumulations over time. To track cellular and sub-cellular events in identified individual animals, we developed a polydimethylsiloxane (PDMS) microfluidic device to grow *C. elegans*, completely immobilize animals in the same orientation, and image the same individual neuronal process throughout development.

Here, we describe the design and optimization of our microfluidic device and demonstrate its utility in capturing high-resolution fluorescence images of the same posterior lateral TRNs across 36 hours (hr) of *C. elegans* development. Mitochondrial distribution along the neuronal process changes over time, however, the density of mitochondria was similar across different developmental stages. Our data suggest that maintenance of density likely occurs through the addition of a new mitochondrion between two mitochondria that are separated by at least 24 μm. Our device can also be used to study other events such as the accumulation of synaptic vesicles (SVs) at the synapses, occurring over several days.
Materials and Methods

Growth and imaging device fabrication

Our microfluidic device was fabricated using soft lithography of PDMS from SU8 resist features on silicon substrates (Whitesides et al., 2001). The device design consists of two photomasks designed in Clewin software and printed using a high-resolution laser plotter (Fine Line Imaging, USA). The flow layer is comprised of a single 10 mm long and 300 μm wide straight channel with one inlet and one outlet reservoir. The control layer had two independently controlled membranes, through one central wide ‘trapping’ channel and two narrow ‘isolating’ channels. The ‘trapping’ membrane is 2 mm wide to immobilize animals while the two interconnected isolation membranes are 300 μm wide to keep the worm within the restricted area of the flow channel. The circular pads for punches and fluidic connections are 2 mm in diameter. Two photomasks were used to produce SU8 masters using UV photolithography for the flow and control layers. The flow layer was fabricated using SU8-2025 (or SU8-2050) spun at 500 rpm for 5 s and 2,000 rpm for 30 s to obtain thicknesses of ~40 μm (or ~80 μm). The control layer was created using SU8-2050 spun at 500 rpm for 5 s and 2,000 rpm for 30 s to produce a thickness of ~80 μm. PDMS (10:1) was prepared by mixing the PDMS base with the curing agent. The PDMS mix was spin coated on the flow layer at 1,000 rpm for 35 s to produce ~120 μm thickness on the SU8 master and baked at 70 °C for 2 hr. PDMS (10:1) was poured on the SU8 pattern for a 5 mm thick PDMS mold for the control layer and baked at 70 °C for 2 hr. The baked control layer was cut and removed from the SU8 master. Access holes were punched for ‘trapping’ and ‘isolating’ channels and bonded on top of the flow layer using 18 W air plasma for 2 min. The bonded block was finally removed from the silicon substrate, holes were
punched through for both inlet and outlet access, and bonded to a cover glass of thickness 170 μm.

C. elegans strains

We used the C. elegans transgenic strains: jsIs821 [mec-7p::GFP::RAB-3] (Kumar et al., 2010) and jsIs609 [mec-7p::MLS::GFP] (Fatouros et al., 2012, Sure et al., 2018) in this study. The strain jsIs821 expressed GFP::RAB-3, marking synaptic vesicles of the six touch receptor neurons. For time-lapse mitochondria imaging, we used jsIs609 strain expressing GFP targeted to the mitochondrial matrix in the six touch receptor neurons. Strains were maintained on NGM plates using standard protocols (Brenner, 1974). Eggs were transferred to fresh NGM plates and allowed to hatch for two hr at 22 °C. Long-term imaging and mitochondrial transport parameters were calculated at multiple time points up to 36 hr after worms were placed in the device.

C. elegans maintenance inside PDMS device

The flow layer of the device was filled with M9 buffer through the inlet and outlet. The two channels in the control layer were preconditioned with a buffer column under 14 psi pressure of nitrogen gas. When pressurized with compressed nitrogen gas, the column of liquid did not leak into the flow channel due to the presence of the PDMS membrane. A single egg or an early larval stage animal, growing on NGM plates, was pushed into the flow channel within the region between the two ‘isolating’ channels. The two ‘isolating’ membranes were turned on while the central ‘trapping’ membrane was left free. The OP50 bacteria culture was diluted in S medium (1×10⁸ cells/mL), filled with 200 μL micro tips, and connected to the inlet of the flow channel.
The outlet was connected with the second pipette tip with a lower food volume compared to the first pipette tip at the inlet. The difference in the height of the food solutions connected to the inlet and outlet channels maintains a constant flow through the flow channel. The small overlap in an area between the ‘isolating’ channels and the flow channel creates a partially closed membrane in the flow channel. The partially closed channel allowed bacteria to flow along the side of the walls of the flow channel while preventing the animal from crossing the ‘isolating’ membranes. A wide central ‘trapping’ membrane sealed the flow channel completely under similar gas pressure and immobilized animals completely for high-resolution imaging.

Measuring body length and diameter

Individual animals growing inside the microfluidic devices and on NGM plates were imaged using a brightfield microscope (Olympus IX71) at 4× and 10× magnification. Images were loaded in Fiji (http://fiji.sc/Fiji) and individual worms, with dark features clearly visible on a light background, were traced with segmented lines. The segmented line was drawn from head to tail along the middle of the worm body to measure and quantify body length. The diameter was approximated with a straight line across the body in the vulva region as the maximum body width.

Time-lapse imaging of C. elegans neurons

C. elegans growing on NGM plates were immobilized using either 3 mM levamisole on 2% agarose pads or without any anesthetic inside the microfluidic device under the ‘trapping’ membrane for high-resolution fluorescence imaging. To count the total number of mitochondria at different developmental stages, we immobilized animals at different stages on...
an agarose pad using 3 mM sodium azide (Sigma-Aldrich) and imaged them within 10 min after
the application of the anesthetic. Time-lapse fluorescence images were acquired using a 60×
(oil immersion and the numerical aperture of 1.4) objective on an inverted microscope
(Olympus IX81) equipped with a spinning disc unit (CSU Yokogawa) and a CCD-based camera
(iXon, Andor) to visualize GFP fluorescence from *C. elegans* neurons. Long-term imaging during
development was carried out on the same neuron using multiple images of the same neuron at
different time points. To maintain animal physiology during long-term imaging experiments,
animals were left free inside the flow channel in the presence of sufficient bacteria between
successive imaging sessions. To estimate synapse growth, GFP::RAB-3 fluorescence was
collected from the ventral synapses of the PLM neurons in device immobilized or 3 mM
levamisole anesthetized animals at different stages of their development.

**Bleach and recovery of stationary mitochondria**

*C. elegans* were mounted on a glass slide on a 2% agarose pad and anesthetized with 3 mM
levamisole (Sigma-Aldrich) for live imaging. An Olympus IX83 inverted microscope attached
with a spinning disc (Perkin Elmer Ultraview) was used to acquire time-lapse images of the 60
μm length of the initial (first 1/3rd from the cell body) or middle (2/3rd from the cell body) of
the PLM neuronal process at either 3 or 4 fps (frames per second) using a 60×, 1.4NA oil
immersion objective. The Andor (iXon DU897-UVB)/Hamamatsu (SZK) monochrome camera
was used to capture transport movies from GFP-labeled mitochondria for 20 min using a 488
nm excitation laser at ~8% transmission. At least 100 frames (25 s) of time-lapse movies were
acquired to identify stationary mitochondria before bleaching one of the stationary
mitochondria in the frame using a 405 nm laser at 60% transmission for 100 ms, 49 iterations of
10 ms each. Kymographs of the simultaneous bleaching and imaging movies were used to identify all the stationary bleached, stationary unbleached, and mobile mitochondria.

Image analysis and quantitation

The physical dimensions of the *C. elegans* body were quantified using brightfield images of animals growing on NGM plates, in 96-well plate liquid cultures, and inside microfluidic devices. Fluorescence images were analyzed in Fiji. For mitochondrial density measurements, multiple image frames covering the entire TRN processes were opened using Fiji. Guided by the features and faint GFP signal present in the neuronal process, the length of the neuronal process was traced between successive mitochondria pairs using both the segmented and freehand line tools in Fiji. Due to linear geometry and absence of 3D curvature in the PLM neuronal process, both tools provided very similar length measurements. Hence, we used the segmented line tool for all our analyses. Every mitochondrial position and intermitochondrial intervals were recorded for every individual TRN at specified time points. Numerous total mitochondrial fluorescence envelopes were counted in a given neuronal process from the cell body to the distal tip of the process at each time point. Each fluorescence envelope was segmented to cover all the bright pixels to estimate the average intensity and total area for each mitochondrion. Intermitochondrial distances were quantified in pixels as distances between the centroid of two successive mitochondria in a neuronal process and converted to micrometers. For long-term mitochondrial analysis, the length of the neuronal process every 3 hr was measured and plotted for an individual animal. A linear regression line was fitted to estimate the growth rate for each animal. Assuming a uniform growth rate, the length of the neuronal process at every 3 hr time point was normalized to match the estimated value, the position of...
all mitochondria was corrected to match the total estimated neuronal process, and new intermitochondrial distances were calculated for further analyses.

Fast time-lapse fluorescence images of the mitochondria were converted to kymographs using the kymograph plugin in Fiji to quantify mitochondrial dynamics along the neuronal process. Kymographs were analyzed for stationary and moving mitochondria using line tracing. The anterograde and retrograde movements were determined using a minimum displacement cutoff of 3 pixels (0.6 μm) over 5 consecutive frames (1.67 s) and a velocity cutoff of >0.01 μm/s. Flux was calculated, using the total number of moving mitochondria normalized to the length of the movie and the entire visible neuronal process length. The fluorescence-decay profiles of stationary mitochondria were calculated by drawing an envelope around the stationary mitochondria before bleaching and the integrated intensity profiles at all successive frames pre- and post-bleach were quantified. To normalize variation in the background due to fluctuations in the laser intensity, we calculated the background signal from an area similar in size as the bleached mitochondrion within the worm but away from the neuronal process and subtracted it from the integrated values. The subtracted intensity profile was used to calculate the percentage drop and the photobleaching time for each stationary bleached mitochondrion. To calculate flux across bleached and unbleached mitochondria, anterogradely and retrogradely moving mitochondria were identified at locations with stationary mitochondria and normalized to the total time elapsed during each movie.

The size of synaptic vesicle accumulation at ventral synapses was calculated from the total number of pixels present inside the fluorescence envelope, consisting of all the pixels with fluorescence intensities higher than the background.
Statistical analysis

Data are presented as the mean ± standard deviation (or standard error of the mean). Histograms are used to show the intermitochondrial distances, gain insight into the shape and variability present in the data, and estimate probability distributions from the experimental values. We used the Freedman-Diaconis rule to estimate the bin size from the interquartile range ($IQR$) and the number of ($n$) intermitochondrial observations (Freedman and Diaconis, 1981). The bin width was estimated using $\text{width} = \frac{2 \times IQR}{\sqrt[n]{n}}$, where the $IQR$ is defined as the difference between the third and the first quartiles. To determine whether mitochondria are distributed uniformly, randomly, or clustered along the neuronal processes, we divided the neuronal processes into 20 μm bins and counted the number of mitochondria in each bin. The number of mitochondria per bin, the standard deviation of the mean, and variance ($\sigma^2$) were calculated. We calculated the index of dispersion ($\frac{\sigma^2}{\mu}$) for mitochondrial distribution along the neuronal processes at t=0 hr (L4) and t=24 hr (D1) stage animals. Based on the index of dispersion, we determined if mitochondria were distributed uniformly ($\mu > \sigma^2$), randomly ($\mu = \sigma^2$), or clustered ($\mu < \sigma^2$). To test the goodness of fit, we used the $\chi^2$ analysis against a Poisson distribution (Miller and Sheetz, 2004).

The normality for experimental observations was calculated using the Kolmogorov-Smirnov test and statistical significance was calculated using either the two-sample t-test or one-way ANOVA (for >2 conditions). The $p$-values <0.05 (*), <0.005 (**), and >0.5 (ns, not significant) was used to assess statistical significance.
Results

Mitochondrial number along the neuronal process increases during development in *C. elegans* TRNs

Mitochondria are essential organelles involved in energy metabolism and play a vital role in diverse biological processes such as aging and apoptosis (Sheng, 2014, Seervi and Xue, 2015, Sun et al., 2016, Melentijevic et al., 2017). Transport and distribution of mitochondria in neuronal processes and at synapses are critical for the normal physiology of neurons (Morsci et al., 2016, Cai and Tammineni, 2017). Since *C. elegans* are transparent throughout their development, it is feasible to track fluorescently labeled organelles such as mitochondria and SVs in TRNs using high-resolution imaging. *C. elegans* TRNs are a suitable model for high-resolution *in vivo* organelle imaging due to its long and planar neuronal process along the body length present close to the cuticle (Fig. 1A, B).

To quantify mitochondrial density in *C. elegans* TRNs, we immobilized *jsIs609* animals and imaged the TRNs using mitochondrial matrix targeted GFP expressed in six TRNs for a single time-point analysis during their development (Fatouros et al., 2012, Sure et al., 2018). We immobilized the animals on a 2% agarose pad using 3 mM sodium azide and counted mitochondria number, within 10 min after the application of the anesthetic, using mitochondrial GFP and length of the neuronal process visible due to some GFP that fills the neuronal process in the posterior lateral mechanosensory (PLM) neurons (Fig. 1C). An increase in neuronal process length has been shown to correlate with an increase in mitochondria number as the animal ages and across developmental stages (Morsci et al., 2016, Awasthi et al., 2020). We found the neuronal process length increases from ~100 μm in early larval stage 1.
animals (L1) to nearly 500 μm in 1 day adult (1D adult) with a concomitant increase in mitochondria number from approximately 6 to 26 in the posterior TRN (Fig. 1D). The bulk of the increase in the length of the neuronal process occurs as the animal grows and occurs after synapse formation at the early L1 stage of development. The addition of new mitochondria maintains a mitochondrial density of ~5 mitochondria/100 μm along the neuronal process length throughout development (Fig. 1E). This density is similar to earlier reports of density measured in adult animals for this neuron (Morsci et al., 2016, Sure et al., 2018). From the above values, the increase in mitochondrial number during development is approximated to ~6 mitochondria every 24 hr (averaging ~1 mitochondrion every 4 hr). The area of mitochondria fluorescence in L1, L4, and 1D adult stages of the animal is measured to vary between 0.05-0.95 μm², 0.05-1.66 μm², and 0.05-2.14 μm², respectively (Fig. 1F). Young larval animals have a greater number of smaller mitochondria compared to later larval stages or adult animals. Single time point images of C. elegans populations of different developmental stages indicate a steady increase in the total number of mitochondria but fail to indicate the location of such addition. To capture individual mitochondrion addition events such that a similar mitochondria density is maintained over development, the same neuronal process needs to be imaged over days under optimal growth and imaging conditions. Since mitochondria are susceptible to cellular stresses (Hill and Van Remmen, 2014, Jovaisaite et al., 2014, Labbadia et al., 2017), it is impossible to image the same animal by repeatedly anesthetizing it over a prolonged growth period. To reduce the adverse effects of anesthetics and provide a physiological environment, we developed a long-term growth and imaging microfluidic platform.
Microfluidic device facilitates growth and high-resolution imaging of \textit{C. elegans}

To study the long-term development of individual \textit{C. elegans} while carrying out high-resolution imaging, we developed a microfluidic device to mimic a physiological environment by supplying constant food where we can immobilize the animal under a deformable PDMS membrane as needed. To make this microfluidic technology easily accessible, we developed a device with a simple design, few fabrication steps, easy operation, no complex valves, using inexpensive accessories. The PDMS device is bonded on a thin cover glass to facilitate high-resolution imaging on an inverted microscope using lenses of various magnifications up to a 100× oil objective.

The device is fabricated as two separate PDMS layers and irreversibly bonded together (Fig. 2A-F). The device utilizes PDMS membrane deflections to isolate and immobilize a \textit{C. elegans} hermaphrodite within the flow channel. In this device, \textit{C. elegans} swim freely and grow in a 300 μm wide flow channel in the presence of bacteria. A pair of partially closed membranes above the symmetric ‘isolating’ channels allows the bacterial suspension to pass through while preventing an individual animal from escaping the flow channel. The individual animal is repeatedly immobilized under a deflected ‘immobilizing’ PDMS membrane. \textit{In vivo} cellular processes are imaged in the animal immobilized beneath the ‘trapping’ channel.

Individual animals of the appropriate developmental stage were picked from an NGM plate, using 5 μL M9 buffer, and inserted inside the flow channel in the absence of immobilization or isolation pressure. The animal inside the flow channel was observed using either a 4× or a 10× objective on an inverted microscope. The height of the bacterial solution in
the two pipette tips, connected at the inlet and outlet, generated sufficient hydrodynamic flow rates to allow bacterial passage through the flow channel to feed the worm (Fig. 2C, F). The deflected membrane in the ‘isolating’ channel restricts animal motion between the two ‘isolating’ channels. Freely moving worms were imaged in brightfield to measure their physical parameters and assess animal health. Animals were completely immobilized in the flow channel under the PDMS membrane using 14 psi pressure in the ‘trapping’ channel. Complete immobilization was required for high-resolution time-lapse imaging of the sub-cellular events in the neuronal processes. Animals were immobilized repeatedly in a straight posture along the flow channel sidewalls under the trapping membrane to bring the same posterior lateral TRN under the best focus for imaging the sub-cellular events (Fig. 3A). Identical animal orientation enabled us to image mitochondrial distribution from the same individual neuron over multiple time points using an inverted confocal microscope equipped with a 60× oil objective (Numerical Aperture 1.4).

Growth in a microfluidic device does not impede *C. elegans* development

To obtain high-quality time-lapse movies of the sub-cellular events during *C. elegans* development, the health of the animal grown in a microfluidic environment during the entire period should be similar to that of an animal grown on standard NGM solid media. *C. elegans* growth in liquid medium with *E. coli* bacteria has been studied (Keil et al., 2016, Solis and Petrascheck, 2011). *C. elegans* were found to develop ~15% slower in liquid culture with adequate bacterial supply nonetheless recapitulated all long-term developmental processes such as molting and egg laying (Keil et al., 2016). Since food supply is one critical component for
normal development, we ensured sufficient bacterial supply by adjusting both the concentration of bacteria and the relative amounts of buffer solution with bacteria in the two pipette tips attached to the channel entrances. The flow channel containing liquid OP50 showed no contamination over 84 hr (4 days) of *C. elegans* growth (Fig. 2G). The pipette tips can be easily replaced with new sterile tips freshly filled with OP50 bacteria as needed.

To characterize *C. elegans* development in our microfluidic device, we measured the body length and diameter of the animals grown in our microfluidic chamber and compared them to values obtained from animals grown on NGM solid media. Our data show that animals growing in the microfluidic device are on average shorter and thinner compared to those grown on solid NGM media (Fig. 2H, I). The body features (body length and diameter) of the animals grown inside the microfluidic device lags by 2 – 3 hr compared to the values obtained from those grown on NGM solid media. These data are similar to those reported earlier for *C. elegans* grown in liquid media (Keil et al., 2016, Solis and Petrascheck, 2011). A hermaphrodite grown inside the microfluidic device laid eggs after 84 hr, that hatch into larvae.

**Microfluidic device facilitates repeated immobilization and time-lapse imaging of the identical neuronal process in an individual animal**

Mitochondria in the neuronal process display distinct transport characteristics; characteristic velocities away from (anterograde) and towards (retrograde) the cell body, intermittent pauses, and bi-directional movement (O’Toole et al., 2008, Cai and Sheng, 2009, Schwarz, 2013, Welte, 2004, Hancock, 2014, Sure et al., 2018). To quantify *in vivo* mitochondrial transport characteristics from the *C. elegans* TRN processes, we immobilized animals in the flow channel
using 14 psi on the trapping PDMS membrane (Fig. 3A). A slow progressive immobilization allows animals to be laterally oriented, facilitating high-resolution imaging of PLM neurons.

To assess the effect of photobleaching and changes in auto-fluorescence due to repeated immobilization, we repeatedly immobilized *C. elegans* at two different time intervals; either trapping every 1 hr or trapping every 6 hr under the ‘immobilizing’ membrane up to a total duration of 12 hr. Under both paradigms, the same PLM neuron (either L or R) of the animal was imaged only at the 0, 6, and 12 hr time points. The ratio of the mitochondrial fluorescence to the background fluorescence (M/B ratio) was calculated at different time points. The decrease in this fluorescence ratio in both paradigms was statistically insignificant (2.07 ± 0.106, 1.80 ± 0.192, *p* = 0.06) (Fig. 3-1A–C). Using repeated imaging of *C. elegans* in our microfluidic device, we were able to obtain a good fluorescence signal from mitochondria compared to the background autofluorescence.

We further characterized the effect of immobilization of an individual *C. elegans* on the variability in the position of each stationary mitochondrion that arises just from the immobilization process and small changes in the posture of the animal. Positions of mitochondria were used to calculate intermitochondrial distances and estimate local compression and expansion of the neuronal process between multiple adjacent mitochondrial pairs. We, therefore, imaged the same TRNs every 5 min for 15 min by repeatedly trapping the animal under the ‘immobilizing’ PDMS membrane and calculated intermitochondrial intervals for each immobilization. Intermitochondrial distance values (L) between identical mitochondrial pairs from the same TRN processes imaged at different time points were compared (∆L). This comparison allows estimation of how the extent of expansion or compression between each
mitochondria pair ($\Delta L/L$) affects adjacent inter-mitochondrial distances. Only stationary bright mitochondria were considered for this calculation. Most mitochondrial positions remain largely unchanged and intermitochondrial distances were used to measure the compression or expansion percentages between each pair of mitochondria (Fig. 3-1D, E). The compression and expansion percentages were distributed symmetrically and represented as positive (compression) and negative (expansion) values, respectively. The $\Delta L/L$ values were distributed around +1.79 % and -2.01 % (2.4 \pm 0.17 \mu m and 2.4 \pm 0.19 \mu m, mean \pm SEM) (Fig. 3-1E). Thus, the errors arising from compression and expansion due to immobilization do not significantly alter the measurement of intermitochondrial distances.

Device-immobilized animals show lower mitochondria flux and turnover compared to anesthetic immobilized animals

We examined mitochondria numbers and their dynamics in NGM grown and device grown animals. The density of mitochondria in larval stages and 1D adult animals grown in NGM and microfluidic devices are similar. Additionally, the inter-mitochondrial intervals in NGM grown animals and device grown animals are very similar. To examine the movement and turnover of mitochondria from both device and anesthetic immobilized animals, we carried out high-resolution time-lapse imaging of TRNs. The acquired movies were converted into kymographs to analyze mitochondrial transport and positions (Fig. 3B, C). Device immobilized animals regained normal body movements within 10 min of release from pressure. Mitochondria are classified as stationary if their displacement is less than 3 pixels (0.6 \mu m) over 5 consecutive frames (1.67 s) or their velocity is <0.01 \mu m/s. A mitochondrion is considered as moving if its
displacement is $\geq 3$ pixels over 5 consecutive frames. Mitochondria move anterogradely away from the cell body or retrogradely towards the cell body. The movement close to the cell body was captured for 20 min at 3 fps to quantify all anterograde and retrograde events in both anesthetized animals and device immobilized L4 stage animals, both grown on NGM plates until imaged. The average number of mitochondria entering the neuronal process from the cell body was about twice the total number of mitochondrial exit events into the cell body (Fig. 3D). The average number of entry and exit events was lower but not statistically different ($p$-value $> 0.5$) in the device immobilized animals compared to anesthetized animals. In device immobilized animals, the rate of mitochondrial entry into the process is twice that of mitochondrial exit as well (Fig. 3D).

The average instantaneous velocity of moving mitochondria in both the anterograde and retrograde directions was slightly higher in the device ($0.40 \pm 0.042 \mu m/s$ and $0.55 \pm 0.098 \mu m/s$) compared to anesthetized immobilized animals ($0.29 \pm 0.011 \mu m/s$ and $0.40 \pm 0.031 \mu m/s$), although statistically significantly different only for the average anterograde velocities (Fig. 3E).

The average anterograde mitochondria flux value was higher than that of the retrograde flux in both device and anesthetic immobilized animals (Fig. 3F). The anterograde and retrograde fluxes in the levamisole immobilized animals ($32.6 \pm 6.70$ and $21.6 \pm 7.40$ mitochondria/hr-100 $\mu m$) were higher as compared to that in the device immobilized animals ($20.8 \pm 6.28$ and $8.0 \pm 2.74$ mitochondria/hr-100 $\mu m$) although the differences are not statistically significant ($p$-value $= 0.20$ and $0.10$). We examined if the greater mitochondrial transport in levamisole (3mM Lev) treated worms, compared to worms immobilized in the device without chemicals (Device) was due to the anesthetic. We acquired time-lapse images of PLM neurons from animals both
anesthetized in 3 mM levamisole and trapped in our microfluidic device (Device + Lev). The ratio of all moving mitochondria to the total number of mitochondria in levamisole immobilized (0.44 ± 0.056, n=18) animals were statistically significantly higher (p-value = 0.004) than in device immobilized (0.22 ± 0.032, n=16) animals (Fig. 3G). The ratio of moving to stationary mitochondria continued to remain high (0.42 ± 0.039, n=14) in levamisole anesthetized animals mechanically trapped by a PDMS membrane (Fig. 3G). Similar effects by anesthetics on neuronal transport flux of vesicular cargo have been reported (Mondal et al., 2011)

Using fast time-lapse imaging of the individual TRNs, at a speed of 3 or 4 fps for a total of 1,000 frames (time ≥ 150 s), we analyzed turnover rates of moving mitochondria across photobleached stationary mitochondria in the middle of the neuron. The stationary mitochondria drop fluorescence intensity by 96.5 ± 0.89% (from n = 20 bleaching events) within 0.33 s, the interval between successive frames at our acquisition rate of 3 fps (Fig. 3-2A-C). Upon photobleaching a stationary mitochondrion, the motion of a small and faint moving mitochondrion can be easily tracked across the site of the photobleached stationary mitochondrion. In a simple cross event, a small mitochondrion moves across the photobleached mitochondrion without contributing fluorescence to the stationary mitochondrion (Fig. 3H, 3-2D and Movie 1). While moving mitochondria are identified as contributing to a photobleached stationary mitochondrion by either pausing and/or splitting a portion of their fluorescence leading to fluorescence recovery at the site of the large photobleached stationary mitochondrion (Fig. 3-2E-G and Movies 2-3). Sometimes paused mitochondria filled the entire region of the stationary mitochondria area before photobleaching and sometimes they merely pause and then continue to move. Both anterograde and retrograde moving mitochondria were
found to contribute to the turnover of stationary mitochondria. On average, more events were observed in anesthetized animals compared to device-immobilized animals across any given bleached mitochondria, likely due to the overall increase in flux in levamisole anesthetized animals (Fig. 3I-J). Both the device and levamisole immobilized animals show a higher number of anterograde movements across stationary bleached mitochondria as compared to retrograde movements, similar to that observed in movies without bleaching (Fig. 3E-F, Fig. 3-2H). We also measured total flux as well as a proportion of events in both the initial 1/3rd (Init) and the middle (Mid) of the neuron (Fig. 3K-L). We see the proportion of moving mitochondria is greater in the levamisole immobilized animal in every region of the neuron image (Fig. 3M). This might account for the small differences in the number of events where a moving mitochondrion interacts with the stationary bleached mitochondria.

We did not observe any stationary mitochondria split into smaller mitochondria in any of our movies during the entire 4.6 hr of imaging either in anesthetic or device immobilized animals. The fluorescence envelopes of moving mitochondria were smaller than stationary mitochondria. We sometimes observed tubular-shaped moving mitochondria in the first 60 μm of the neuronal process proximal to the cell body. Anesthetic immobilization effects perhaps account for the increase in mitochondrial movement which in turn may lead to a higher turnover of mitochondria along the neuronal process.

**Mitochondria are added along the entire C. elegans TRN neuronal process**

Mitochondria are added to a growing neuron after synapse formation to maintain a constant mitochondrial density (Morris and Hollenbeck, 1993). For example, the number of mitochondria
and total process length respectively increased from 7.8 ± 0.41 and 125 ± 6.07 μm in L2 larvae (n = 15 animals) to 20.1 ± 1.44 and 335 ± 15.89 μm in L4 larvae (n = 10 animals, p-value < 0.01) in the microfluidic device. The neuronal process grows at the rate of ~10 μm/hr and the mitochondria number increases ~0.6 mitochondria/hr, numbers very similar to those seen in animals grown on NGM plates (see above). Although the population statistics indicate average growth rates, it does not indicate the site where new mitochondria are added.

To identify the location where the new mitochondria are added as the animal is growing, we imaged the same PLM neuron every 3 hr for approximately 36 hr by repeatedly trapping the animal in our microfluidic device. The images were used to reconstruct the neuron and the location of each mitochondrion was plotted for every imaged time point. The total mitochondria number increased linearly with imaging time. Both, total mitochondria number and neuronal process length at 24 hr were significantly higher than at the initial time point (Fig. 4A). We chose the cell body, branch point, and the neuronal process tip as the fiduciary markers to examine if there was any bias in the growth of the neuronal process as new mitochondria are added along the neuronal process (Fig. 4E). The ratio of the neuronal process lengths between the cell body to the branch point and the branch point to the neuronal process tip remains constant as the neuron and the animal grows suggesting proportional growth along the neuronal process (Fig. 4B-C). Over 36 hr of imaging, the average total neuronal process growth rate was calculated to be ~9 μm/hr (n = 8 animals). We calculated the ratio of the mitochondria number present along the neuronal process between the cell body and the branch point to the mitochondria number between the branch point and the neuronal tip. The
ratio of the mitochondria number remains constant over a 24 hr period (Fig. 4B), which suggests that new mitochondria are likely added uniformly along the process length.

To capture the position and size of every mitochondrion, we reconstructed the entire neuronal process from a series of fluorescence images at multiple time points and analyzed various parameters such as mitochondrial number, size, and the intermitochondrial intervals between each adjacent pair of mitochondria. Approximately 20% of mitochondria move in any given frame in the TRNs with an average velocity of approximately 200 nm/s (Fatouros et al., 2012). Moving mitochondria are less than ~1 μm in diameter. Stationary mitochondria are bigger in size but drift gradually over time as the animal grows and the neuronal process commensurately proportionately elongates. Small moving mitochondria have low fluorescence intensity and appear randomly and intermittently along the TRN processes in our long-term images due to large time intervals between successive frames. The size of the stationary mitochondria in the neuronal process remains unchanged over longer time scales i.e. 24 hr (Fig. 4D). We consider a pair of mitochondria to be identical and stationary if the position of each mitochondrion from the cell body, branch point, and tip of the neuron changes proportionately to the average growth rate of the neuron for that animal (typically ~27 μm per 3 hr). The neuronal processes of each individual TRN at multiple time-points are aligned with each other from the cell body where the cell body, the branch point (BP), and the neuronal process tip act as reference points (Fig. 4F). With the growth of the neuronal process, the fiduciary markers (BP and tip) and all the mitochondria move further away from the cell body with time (Fig. 4-1A). For every mitochondrion, the ratio of the new position to the old position was 1.1 ($L'_i / L_i$ where, $L'_i$ and $L_i$ are the new and old position of the $i^{th}$ mitochondria with respect to the cell body).
correlating with the 10% increase in the total neuronal process length over 3 hr. The uniform increase of the neuronal processes and the gradual shift in the stationary mitochondria positions are independent of the alignment of the neuronal process with respect to the cell body or the tip of the neuron (Fig. 4-1B).

To identify a new mitochondrion addition, we identify a mitochondrion between a pair of pre-existing stationary mitochondria with higher fluorescence than the background intensity from the neuronal process. The new mitochondrion must stay in the same location for at least two successive frames (i.e. greater than 6 hr). The addition of a new mitochondrion was identified using manual inspection of the relative mitochondrial position and geometrical parameters (size and integrated intensity) of the new mitochondrion after aligning the axons with respect to the fiduciary markers (Fig. 4-1).

The number of mitochondria addition events was calculated for every equal third of the neuronal process i.e. initial, middle, and end. The number of events in each third of the neuron was similar 33.3 ± 3.24% (n = 29), 37.5 ± 2.61% (n = 32), and 29.1 ± 3.31% (n = 24) with p ≥ 0.07 (Fig. 4-2A). We see that each new mitochondrion added has an approximate size of 0.47 ± 0.07 μm² (mean ± SEM) when first identified and grew to 0.68 ± 0.11 μm² (n = 4 animals and n = 24 new mitochondria addition events) after 3 hr. The gain in mitochondrial size can come from contributions from either the moving mitochondria or in situ biogenesis. Moving mitochondria along the neuronal processes are likely to initiate a new location and subsequently gain mitochondrial material by fusion-fission of moving mitochondria to form a larger stationary mitochondrion over time in the neuronal process. Using a long-term imaging platform, we were able to identify the addition of new mitochondria in an identified neuronal process.
Increased intermitochondrial distance leads to the addition of a new mitochondrion in \textit{C. elegans} TRNs. The growth in the neuronal process increases the intermitochondrial distance between each adjacent pair of mitochondria. This growth of the neuron occurs along with new mitochondria addition such that density is maintained. To characterize changes in mitochondria density and identify the site of the new mitochondrion addition, we calculated the intermitochondrial distances between each adjacent pair of mitochondria (center-to-center distance along the neuronal process length) in all eight L4 larval animals (Fig. 4-2B). The average distance between adjacent mitochondria was $16.7 \pm 0.96 \, \mu m$ (mean $\pm$ SEM). To determine whether the distribution of mitochondria along the neuronal process was uniform, random, or clustered, we divided the neuronal processes from all 8 animals into 20 $\mu m$ bins and counted the number of mitochondria per bin. The average number of mitochondria per bin = 1.0 with a variance of 1.16 $\pm$ 0.59 (mean $\pm$ variance, n=101 mitochondria); degrees of freedom = 4; $\chi^2 = 10.02$; and a confidence level $P_{(10,02,4)} = 0.04$ suggesting a uniform distribution. Mitochondria can be added at different regions of the neuron altering intermitochondrial distances due to addition of new adjacent mitochondrial pairs (Fig. 4-3). (1) Mitochondria can be added near the cell body, (2) at the end of the neuron, or (3) along the neuronal process when the intermitochondrial distance between adjacent mitochondria increases beyond a threshold. In either possibility (1) or (2) the intermitochondrial distance histograms will show two peaks, one at shorter and another at longer intermitochondrial distance intervals corresponding to additions in one region of the neuron and with increasing distance between other adjacent
mitochondria pairs due to axon growth (Fig. 4-3B-C). In the third possibility, as the mitochondria move apart due to increasing neuronal process length, the entire intermitochondrial distance distribution will shift to higher values (resulting in a low number of events for the short intermitochondrial distance values). In this case, a new mitochondrion will be added in between the old adjacent mitochondria pair and reducing the likelihood of longer intermitochondrial distance values. Hence, we expect to observe a small reduction in shorter-intermitochondrial distances but a relatively unchanged longer-intermitochondrial distance (Fig. 4-3D).

Our data suggest that new mitochondrion addition occurs when the intermitochondrial distance between adjacent mitochondria increases beyond a threshold (orange, Fig. 5A–D). An adjacent pair of mitochondria moving apart by 25 μm, is still below the threshold and does not dock a new mitochondrion in between them (gray, Fig. 5A, B). To study mitochondrial addition in a growing neuronal process, we compared the intermitochondrial distances obtained from images at 0 hr and 24 hr of eight different animals at an early L4 and the 1D adult stages, respectively (Fig. 6A). After 24 hr, the population average of the intermitochondrial distances between adjacent mitochondria increased from 17 ± 0.7 μm to 19 ± 0.6 μm (mean ± SEM, n = 8 animals). To determine if mitochondria addition on the neuronal process, continues to maintain a uniform distribution, we performed a χ² analysis with all 8 animals at the 24 hr time point. We calculated the average number of mitochondria (n=197) per bin=1.0 at 1.02 ± 0.53 (mean ± variance), degrees of freedom = 4, χ² = 23.55, and the confidence level \( P_{(23.55, 4)} = 0.0001 \) suggesting a uniform distribution. This analysis demonstrates that the neuronal processes continue to maintain a uniform distribution of mitochondria, even after multiple additions of
new mitochondria. The addition of new mitochondria over 24 hr causes a more skewed distribution of intermitochondrial distances as compared to the 0 hr time point (skewness and kurtosis measures increased from 0.81 and 0.34 at 0 hr to 0.89 and 0.80 at 24 hr, respectively). The larger values for the skewness and kurtosis measured at 24 hr indicate a strong right-tailed distribution, indicative of non-random mitochondrial positions as the neuronal process grows. Over the same 24 hr period, the average values in the first two bins (% of adjacent mitochondria separated by 3 μm and 6 μm) reduced from 7 ± 1.03 % and 9 ± 1.27 % to 3 ± 1.17 % and 7 ± 1.01 % (p-values = 0.02 and 0.17), respectively. No other bin in the normalized intermitochondrial interval histogram was significantly different at the end of this imaging period. Although, lengthening of the neuronal process results in mitochondria moving apart (as indicated by the significant reduction in the shorter inter-mitochondrial intervals), the addition of new mitochondria ensures an insignificant increase in the larger intermitochondrial distance values. Although mitochondria undergo dynamic membrane fission/fusion and active transport (Sheng, 2014), their addition and positioning along the TRNs were surprisingly found to be regulated as reflected by the highly skewed distribution of the intermitochondrial distance values.

Adjacent intermitochondrial separation initiates the docking of a new mitochondrion forming two new adjacent independent pairs of mitochondria. One pair (Pair#1A) is with the old mitochondria close to the cell body and second pair (Pair#1B) is with the old mitochondria far from the cell body (Fig. 6-1A, B). The intermitochondrial distances between the old mitochondrial pair at the imaging time before a new mitochondrion is added show a distribution with an average interval of 34 ± 1.0 μm (mean ± SEM, n = 85 mitochondria addition
We identified all new mitochondria pairs to estimate the average distance for Pair#1A and Pair#1B as $17.1 \pm 0.8 \mu m$ and $21 \pm 1.2 \mu m$ (mean ± SEM), respectively for all 85 events (Fig. 6-1D). The new mitochondria addition events are uniformly distributed along the neuron (Fig. 6-1E). Consistent with the third possibility (Fig. 4-3D) we observed 85 events where two adjacent mitochondria had an inter-mitochondrial interval >24 $\mu m$ when a new mitochondrion position appeared and remained for 6 or more hours. The addition of new mitochondria occurs uniformly throughout the neuron and maintains a similar average intermitochondrial distance with its adjacent mitochondria over a day of long-term imaging. Mitochondria positions can be estimated from the experimental probability distribution function. Using all the individual events where a new mitochondrion was added, we calculated the experimental probability distribution of new addition events and predicted possible locations for the addition of new mitochondria using this probability distribution. The normalized plot of intermitochondrial distance (Fig. 6B) gives us the experimental probability distribution with a sum of all the values ($\Sigma P = 100\%$). For any mitochondrial pair, we calculate the intermitochondrial distance ($x = L$) and add normalized numbers of mitochondrial addition for all the intermitochondrial distances lower than the current value to assign the probability ($P_L$) for the addition of a new mitochondrion between a given adjacent pair ($P_L = \Sigma_{i=1}^{L} P_i$, where $i \leq L$). To test our experimental probability distribution values, we imaged a neuronal process length ($L_0 = 186.7 \mu m$) with total mitochondria ($N_0 = 12$) at time $t = 0$ hr and predict that the
hypothesised neuronal process grows to a length \( L_{33} = 573.7 \, \mu \text{m} \) at time \( t = 33 \, \text{hr} \) after the start of imaging. This gave us 12 images that were collected every 3 hr (Fig. 6C). For the ‘predicted’ neuron, we calculate that the neuronal process elongates steadily with an experimental rate of 11 \( \mu \text{m/hr} \), similar to the mean neuronal process elongation rate of \( \sim 9 \, \mu \text{m/hr} \). Using the above parameters, we estimate that the neuronal process (with an average intramitochondrial distance of \( 17 \pm 2.8 \, \mu \text{m} \) at \( t = 0 \, \text{hr} \)) is predicted to grow \( \sim 11 \, \mu \text{m/hr} \) in 33 hr leading to an average intermitochondrial interval of \( 52 \pm 8.7 \, \mu \text{m} \), without the addition of new mitochondria (Fig. 6C). When the separation between adjacent mitochondrial pairs increases beyond 24 \( \mu \text{m} \), we predict the addition of a new mitochondrion (Fig. 6B). This prediction is supported by our experimental observations (Fig. 6C). The addition of a new mitochondrion between two stationary mitochondria, as the neuronal process expands with the growth of an animal, is controlled by the rate of neuronal elongation, a gradual increase in the intermitochondrial distances beyond the 24 \( \mu \text{m} \) threshold, and predicted by the experimental probability distribution.

This technology allows us, for the first time, to track individual neurons \emph{in vivo} to assess dynamics that occur over slow timescales such as intracellular organelle positioning across development. Using our long-term imaging device, we were able to extract the experimental probability distribution for organelle addition along the axon and use the values to predict the location of a new mitochondrion addition between a pair of adjacent pre-existing mitochondria in a growing neuron. Multiple factors could determine the addition of new mitochondria such as the ratio of molecular motors (Sure et al., 2018, Chen et al., 2021), local cytoskeleton (Sood et al., 2018), local calcium concentration (Wang and Schwarz, 2009b), new docking sites (Cai et al., 2018).
Our long-term device can be used to study synaptic development in an individual animal

We wanted to test whether our device could be used to image other developmental processes to examine its general utility for other types of biological processes. To perform high-resolution time-lapse imaging of sub-cellular organelle accumulation events in *C. elegans* across developmental stages, we captured fluorescence images of GFP::RAB-3 accumulation at the ventral PLM synapses from the same individual *jsIs821* animals (Kumar et al., 2010, Nonet, 1999, Mahoney et al., 2006). We immobilized each animal using our imaging platform under 14 psi pressure inside the flow channel. Slow progressive immobilization provides a suitable lateral orientation of *C. elegans* and the best view of the PLM synapses (Fig. 7). We immobilized the same animal in a lateral orientation to image both PLM synapses at L2 (16 hr), L3 (22 hr), L4 (38 hr), and 1D adult (68 hr) stages from the time the eggs hatched. High-resolution time-lapse images were analyzed for synapse sizes to estimate synapse growth at different developmental stages. The amount of GFP::RAB-3 accumulation shows a gradual increase similar to the prior average signal measured from multiple populations of *C. elegans* animals immobilized inside the microfluidic device at different developmental stages (Mondal et al., 2011). This increase is thought to arise from the gradual increase in the net anterograde flux of synaptic vesicles (Mondal et al., 2011). Our imaging platform captured a robust increase in the accumulation of GFP::RAB-3 at the synapses of the same animal over multiple developmental stages.
Discussion

*C. elegans* TRNs grow from approximately 100 μm at the L1 larval stage to 500 μm in 1D adult stage. During this growth, approximately 20 new mitochondria are added to maintain a constant mitochondrial density of ~5 mitochondria/100 μm along the neuronal process (Fig. 1D-E). The density measured in adult animals grown in our microfluidic device is very similar to that previously reported in young adult animals (Morsci et al., 2016, Sure et al., 2018). Conventional anesthetic assays can have detrimental effects on animal physiology i.e. it can alter muscle tone consequently altering body length, and cannot easily be used for long-term imaging of the same animal. Anesthetic-free immobilization using a microfluidic device shows robust vesicle transport (Mondal et al., 2011), fast axonal regeneration rates (Guo et al., 2008), and quick behavioral recovery (Manjarrez and Mailler, 2020, Dong et al., 2018). In agreement with our previous vesicle transport studies (Mondal et al., 2011), we found robust mitochondrial transport in the device immobilized animals. To follow slow mitochondria addition during neuronal process growth, we developed a microfluidic device that can be used to grow *C. elegans* throughout its development without significant adverse effects on its growth and health (Fig. 2A-F). Its easy operation enables continuous imaging of the same animal inside the microfluidic environment to quantify *in vivo* sub-cellular process in the neuron throughout development. Mitochondria in the TRNs were found to show lower flux in the microfluidic device compared to anesthetic immobilization (Fig. 3D). These observations are similar to the lower flux of synaptic vesicles when immobilized in microfluidic devices compared to anesthetic immobilization (Mondal et al., 2011). Using long-term imaging over a period of 36 hr, we identified mitochondria addition and changes in intermitochondrial distances in *C. elegans* TRNs.
during its elongation after synapse formation (Fig. 4F). Using our microfluidic device platform, one can monitor slow processes such as the addition of mitochondria. Slow entry and exit and limited turn-over of mitochondria identified in our imaging platform suggest that the neuron retains positions of old mitochondria and adds mitochondria at new locations between pre-existing mitochondria along the growing process during development (Fig. 4F).

New mitochondrion addition during growth and axon stretching has been shown to occur between adjacent pairs of stationary mitochondria (Miller and Sheetz, 2004, Misko et al., 2012, Matsumoto et al., 2020, Lamoureux et al., 2010). Our data are consistent with prior observations where we see a uniform distribution of mitochondria, proportionate growth of the neuron, and addition of mitochondria occurring anywhere along the process between two stationary mitochondria (Fig. 5). This mitochondrion addition occurs anywhere along the neuronal process whenever the average separation between two nearest stationary mitochondrial neighbors is at least 24 μm (Fig. 6B), suggesting the presence of a signal that detects intermitochondrial distances.

We have identified the experimental probability distribution for the addition of a new mitochondrion in a growing neuronal process in vivo over a 24 hr period. The distribution for the intermitochondrial distances becomes more skewed and the mean intermitochondrial distance increases with the development of the animal (Fig. 6A). Although new mitochondria are added over the 24 hr time, the index of dispersion indicates that the distribution remains uniform (Fig. 6A table) strengthening the argument that addition is not a random process. As development and growth occur, the number of closely spaced mitochondria is reduced, decreasing the fraction of mitochondria with lower separation between them. During neuronal
process elongation, the new mitochondria added are small in size and have low integrated fluorescence intensity, they increase in size and intensity over time (Fig. 4-1). There are two possible mechanisms for the addition of a new mitochondrion that remains a subject for further studies: 1) a small mitochondrion enters from the cell body, moves along the neuronal process, identifies a large intermitochondrial separation, and anchors within the “mitochondria-in-demand” region and 2) a local mitochondrion fission event causes a new small mitochondrion to emerge from a nearby large mitochondrion, moves a shorter distance, and anchors within the “mitochondria-in-demand” region. However, we have not observed a large stationary mitochondrion show such fission in our imaging. All events appear to be initiated by moving mitochondria undergoing fusion or fission with a pre-existing stationary mitochondrion.

Stationary mitochondria when photobleached using a high-power laser, lose nearly 96.5% of the fluorescence intensity instantaneously (in 0.33 s when captured at 3 fps rate, Fig. 3-2B). We think our bleaching protocol is unlikely to damage mitochondria sufficiently to make them generally permeable to cytoplasmic GFP or GFP coming from another mitochondrion that crosses this bleached stationary mitochondrion. We observe several examples where rapidly moving mitochondria cross photobleached mitochondrion locations without any significant change in fluorescence intensity of the moving or the photobleached mitochondria (Fig. 3-2D-\textit{F}). Only a subset of moving mitochondria interacted with a photobleached mitochondrion.

Halting of a mitochondrion at the new ‘mitochondria-in-demand’ region is likely to be facilitated by several factors such as motors, local calcium levels, local ATP concentrations, cytoskeleton architectures, etc. Thus, the addition of new mitochondria likely arises from the stalling of moving mitochondria at a location, between a pair of previously docked
mitochondria, with a probability that increases with the increasing separation between two adjacent stationary mitochondria. The stalled mitochondria appear to enlarge in size potentially through fusion with material from moving mitochondria that go past it. We believe that time-lapse imaging of adjacent mitochondrial pairs greater than 24 μm apart, identified from our long-term time-lapse imaging platform, could shed light on mechanisms of mitochondrial addition. Time-lapse imaging of fission mutants and animals with transport defects can also help delineate the underlying processes and molecular players important in new mitochondria addition events. The long-term mitochondria dynamics studies in C. elegans models using a microfluidic platform not only offers a robust assay to study organelle transport in vivo, but it provides an opportunity to move beyond simple observations obtained from in vitro assays to discover genes that can regulate the positioning of mitochondria in neurons.

Our device can be used at different time intervals. For studies that require more frequent imaging of the same animal, we immobilize the same animal every hour for 12 hr (Fig. 3-1). A 1-hr more frequent immobilization showed greater but statistically insignificant photobleaching when imaged at 12 hr time point (Fig. 3-1C). Intermittent long-term imaging separated by ~6 hr for 52 hr shows a steady increase in synaptic accumulation of GFP::RAB-3 when measured from an individual animal grown inside the microfluidic device. The amount of accumulation was slightly higher in device-grown animals as compared to animals grown on NGM plates (Fig. 7) (Mondal et al., 2011). This increase compared to animals grown on NGM plates may arise from additional mechanical stimulation provided by the deflected membrane pressing against the animal. Despite these caveats, our microfluidic platform can be utilized for a variety of studies to assess long-term cellular or sub-cellular dynamics in C. elegans. Similar technologies can be
used for other model organisms by modifying the channel geometries (Mondal et al., 2012, Mondal et al., 2011).
Figure Legends

Figure 1. Mitochondria number and process length increase linearly with development. A, Schematic of C. elegans with six touch receptor neurons (TRNs). B, Schematic of posterior lateral mechanosensory (PLM) neuron cell body (CB) with a long major neuronal process with a bifurcation of the process at the branch point (BP) to form the synaptic branch that forms synapses (S) along the ventral cord. The mitochondria are indicated with arrows. C, Image of a PLM neuron with 22 mitochondria along the neuronal process. The schematic below the image shows the positions of all 22 mitochondria in the same neuronal process. The scale bar is 20 μm. D, Mitochondria number scales linearly with neuronal process length. Neuronal process length and number of mitochondria of all four larval stages (L1, L2, L3, and L4) and 1 day adult (1D adult). The trend follows a linear regression with a slope of 4.9 mitochondria in 100 μm neuronal process length (total number of animals = 154). E, Mitochondria density remains constant throughout development. Data represented as mean ± SEM (standard error of mean) for L1 (n = 30), L2 (n = 25), L3 (n = 36), L4 (n= 33), and 1D adult (n = 30). Statistical significance was evaluated using one-way ANOVA with Bonferroni post-hoc comparisons; p-value <0.05 (*) and >0.05 (ns) when compared with L1 stage. F, Histogram of the normalized number of mitochondria of a given size represented as a % in the TRNs of L1, L4, and 1D adult stage of animals (n ≥ 148 mitochondria for each stage).

Figure 2. Growth and development of C. elegans are unaffected in the long-term growth and imaging microfluidic device. A, Schematic of the microfluidic device with a flow channel in the bottom layer (L1, grey) and ‘isolating’ and ‘trapping’ channels in the control layer on top (L2, orange). The ‘isolating’ membrane is always kept on with 14 psi pressure (deflected membrane...
denoted by dark orange) to restrict the free animal shown in the inset. **B**, Trap pressure is turned on (14 psi, deflected membrane denoted by dark orange) to immobilize the animal inside the flow channel (inset) during imaging. **C**, 3D view of the device layout with two pipettes tips (light green) as food inlet and outlet connecting the flow channel (dark green). **D**, Schematic for the flow layer with channel dimensions. **E**, Schematic of the control layer with channel dimensions. **F**, Image of a growth and imaging device connected to compressed nitrogen gas and food supply. **G**, Images of a *C. elegans* hermaphrodite growing inside the microfluidic device at 0, 12, 24, 36, 48, 60, 72, and 84 hr post-hatching. The animal is fed OP50 bacteria and isolated inside the flow channel and imaged with a 4× or 10× objective. The red arrows indicate freshly laid eggs at 84 hr. Scale bars are 50 μm or 100 μm at the 72 and 84 hr time points. **H-I**, jsIs609 animals grown in the device (black) and on regular NGM plates (blue) were used for calculating body length (**H**) and body diameter (**I**). The small inset shows the schematic for body length and body diameter from worm images. The average values are mentioned on top of each bar. Data represented as mean ± SEM, n = 12 animals. Statistical significance was evaluated by paired sample t-test; *p*-value <0.05 (*), *p*-value <0.005 (**), and >0.05 (ns) when compared between animals grown on NGM and in liquid culture.

**Figure 3.** High-resolution transport imaging of GFP-labeled mitochondria in levamisole and device immobilized *C. elegans* neurons. **A**, Image of a free and an immobilized larval 4 (L4) stage *C. elegans* in the flow channel. The schematic shows the initial 60 μm from the cell body and the middle of the neuronal process (120 μm from the branch point towards the cell body) that is imaged at high-resolution. **B**, Montage of six frames of a PLM neuron with GFP-labeled mitochondrial fluorescence from time-lapse imaging acquired using a 60× oil objective (1.4 NA).
C, Kymograph of 400 frames acquired at 2 frames per second (fps) for 200 seconds. Anterogradely moving (red arrow), retrogradely moving (blue arrow), and stationary (black arrow) mitochondria are indicated on the images. D, Number of mitochondria entering the neuronal process (entry) and leaving the process (exit) per hr measured in animals immobilized with 3 mM levamisole (n = 35 animals) and inside the microfluidic device (n = 22 animals). E, The average velocities (both in anterograde and retrograde directions) are shown for animals that are immobilized inside the microfluidic device (n=47 anterograde and n=15 retrograde segments) and compared to animals immobilized with 3 mM levamisole (n=336 anterograde and n=142 retrograde segments). F, The average flux values of moving mitochondria from L4 stage animals immobilized inside the microfluidic device (n=16) and with 3 mM levamisole (n=18). G, The plot showing the ratio of moving mitochondria to the total number of mitochondria for device immobilized (n=16), levamisole immobilized (n=18), and levamisole treated animals that are also immobilized in the device (n=14). H, Kymograph representing an anterograde moving mitochondrion (red arrow) crossing a site of a bleached stationary mitochondrion (black arrow). I, The average anterograde events per hr (cross, pause, and split) are measured at the initial (Init, 60 μm from the cell body) and the middle (Mid, a region 120 μm from the branch point towards the cell body) of the neuronal process (schematic is shown in panel A) for both device (Device, n > 8 animals) and anesthetic (Levamisole, n > 29 animals) immobilized L4 animals. J, The average retrograde events are measured by using the device and anesthetic immobilized animals. K, The anterograde flux are measured from all the moving mitochondria in the device and levamisole immobilized L4 animals from the Init and Mid of the PLM neuronal process. L, The retrograde flux are measured from all the moving mitochondria in
the device and levamisole immobilized L4 animals from Init and Mid neuronal processes. Anterograde and retrograde fluxes are calculated from \( n = 10 \) (Init) and \( n = 10 \) (Mid) animals in the device, while \( n = 16 \) (Init) and \( n = 26 \) (Mid) animals in the levamisole. \( \textbf{M} \), The ratio of moving mitochondria to the total number of mitochondria for the Init and Mid portion of the PLM in the device and levamisole immobilized animals. Data represented as mean ± SEM (\( \textbf{D}, \textbf{E}-\textbf{G}, \textbf{I-M} \)). Statistical significances are evaluated by paired sample t-test (\( \textbf{D-F, I-M} \)) and one-way ANOVA (\( \textbf{G} \)); \( p \)-value <0.05 (*), \( p \)-value <0.005 (**), and \( p \)-value >0.05 (ns) are represented. The scale bars are 100 \( \mu \)m (\( \textbf{A} \)), 10 \( \mu \)m (\( \textbf{B} \) and \( \textbf{H} \)), and 5 \( \mu \)m (\( \textbf{C} \)). The vertical bars are 20 s (\( \textbf{C} \) and \( \textbf{H} \)). Table 2, figures 3-1 and 3-2, and Movies 1-3 support figure 3.

**Figure 4.** Total number of mitochondria increases linearly in TRNs in animals growing inside microfluidic devices. \( \textbf{A} \), The average number of mitochondria and the neuronal process length increases starting at 0 hr (L4) to 24 hr (1D adult) of imaging time inside the microfluidic device. \( \textbf{B} \), The ratio of the mitochondria number and the process length was unchanged over this 24 hr imaging time period. The ratio is calculated for the values from the cell body to the branch point and from the branch point to the neuronal tip. Statistical significance was evaluated by paired sample t-test; \( p \)-value <0.05 (*), \( p \)-value <0.005 (**), and \( p \)-value >0.05 (ns) when comparing between the 0 hr and 24 hr imaging time point data. \( \textbf{C} \), Time course of the mitochondrial number and neuronal process length from 8 animals imaged every 3 hr in microfluidic devices beginning at L4 stage. Data represented as mean ± SEM (\( n=8 \) animals). \( \textbf{D} \), The relative frequency distribution of all mitochondria sizes at 0 and 24 hr imaging time points (\( n=8 \) animals). Statistical significance was evaluated by paired sample t-test (\( p \)-value = 0.92). \( \textbf{E} \), A schematic of the TRN shows the neuronal process with the cell body (CB), the neuronal
process end (Tip, red triangle), and the branch point (BP, blue triangle) as fiduciary markers. The branch point is used to estimate the total mitochondria number and neuronal process length between the cell body and the branch point and between the branch point and the end of the neuronal process. F, The same animal was trapped inside the microfluidic device every 3 hr and mitochondria imaged at high-resolution from the same TRN. Fluorescence images were straightened and raw images are displayed. The fiduciary markers of the cell body, branch point, and neuronal process end are used as references. New mitochondria added are labeled with black triangles and followed in two successive imaging time points and marked using dashed lines. Scale bar 20 μm. The red box and the inset show a section of the neuronal process at 18, 21, and 24 hr time points after start of imaging. A new mitochondrion (black triangle) is added in between the cell body and a stationary mitochondrion (gray triangle). The distance between the cell body and the stationary mitochondria increases with time due to the growth of the neuronal process. Figures 4-1, 4-2, and 4-3 support figure 4.

**Figure 5.** Addition of new mitochondria during long-time imaging of the animal grown in the microfluidic device. A, Map of mitochondrial position from an individual *C. elegans* PLM neuron 36 hr after hatching. Solid lines represent all two locations CB and Tip of the neuronal process. The shaded area in gray (Pair#1) is a region in the neuronal process where no mitochondrion is added between adjacent pairs in 30 hr of imaging. The shaded area in orange (Pair#2) represents regions of the same neuronal process where four new mitochondria are identified between the adjacent pairs at different imaging times. The black arrows represent locations of all four new mitochondria. B, Intermitochondrial intervals at different imaging times of two different pairs of stationary mitochondria (Pair#1 and 2) from an individual TRN process.
arrows indicate the time-points when a new mitochondrion was added between the two adjacent mitochondria along the TRN process. The values indicate the total distance between parent adjacent pairs at the 30th hr of imaging, upon adding all the intermediate intermitochondrial intervals formed due to new mitochondria additions. C, The bar represents the intermitochondrial distances for Pair#2 measured from time-lapse imaging. Upon addition of a new mitochondrion, each pair of mitochondria is labeled as new and represented by two new colors (light and dark). The numbers on each bar indicate the intermitochondrial distances for every mitochondria pair. The shorter intermitochondrial distances in a few time points arise due to local compression/expansion of the neuronal process and are considered to be artifacts of imaging due to animal posture. D, Schematic of the mitochondria lineage in Pair#2. The gray dots on the right represents the original adjacent mitochondria pair (not to the scale) while the new color dot indicates the location of the new mitochondrion added. Whenever a new mitochondrion is added, the intermitochondrial distances between new adjacent pairs are indicated by a line with a color similar to the color of the bars. The inset shows the schematic of a growing PLM neuron during the development of C. elegans. The cell body is on left and the neuronal process end is on right. The inset also shows two mitochondria (M1 and M2) separated by length L1.

Figure 6. Experimental probability distribution can predict the addition of new mitochondria in a growing neuronal process. A, Normalized number of intermitochondrial distances at 0 hr (L4) and 24 hr (1D adult) after the start of imaging. Statistical significance was evaluated by two-sample t-test; p-value <0.05 (*) for the first two bars at bins 3 and 6 μm. The table represents the index of dispersion ($\frac{s^2}{\mu}$) and p-value obtained from the goodness of fit estimated using the $\chi^2$
analysis against a Poisson distribution for data from 0 hr and 24 hr imaging time points (n = 8 animals each). B, Normalized number of mitochondria addition is calculated from experimental long-term images of individual TRNs (n=8 animals started at the L4 stage, 85 new mitochondria additions). The probability of new mitochondria added for intermitochondrial distance x = L is shown as $P_L$ and can be estimated by summing $\left( \sum_{i=1}^{L} P_i \right)$, where $i \leq L$.

C, The predicted (dark circle) and experimental (open circle) location of mitochondria at 0 and 33 hr images of an individual animal. All the 12 positions at t = 33 hr are predicted using the locations measured at t = 0 hr and total neuronal process elongation values. The intermitochondrial distances (gray bars with the values) are used to predict the addition of new mitochondria between each pair at 33 hr. The probability values for finding new mitochondria within the predicted pairs are represented in percentages. Figure 6-1 supports figure 6.

**Figure 7:** High-resolution imaging of GFP::RAB-3 in a developing C. elegans inside the microfluidic device. A, Schematic of PLM neuron with its cell body (CB), its neuronal process, and its ventral synapse (S) at L2 larva and 1D adult stages. B, The plot of synapse sizes at different developmental stages of C. elegans growing inside a microfluidic device and on NGM plates. Data represented as mean ± SEM. Statistical significance was evaluated by one-way ANOVA with Bonferroni post-hoc comparisons; $p$-value <0.05 (*) and $p$-value <0.005 (**) when compared to data from L2 stage; $p$-value <0.05 (+) and $p$-value <0.005 (++) <0.05 (ns) when compared between animals grown in the device and on NGM solid media; nonsignificant comparisons are not indicated. The number of synapses analyzed from animals grown on NGM solid media is $n \geq 37$ and in devices are $n = 18$ (L2), 16 (L3), 14 (L4), and 8 (1D adult). C-D, Images of PLM synapses at L2, L3, L4, and 1D adult stages of development grown in the
microfluidic device (C) and on NGM solid media plates and imaged on agar pads (D). The scale bar is 10 μm.
Table 1: Statistical analysis for different figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Normality</th>
<th>Test used</th>
<th>p value</th>
<th>Power</th>
</tr>
</thead>
<tbody>
<tr>
<td>1E</td>
<td>Yes</td>
<td>One-Way ANOVA with Bonferroni post-hoc</td>
<td>1.000</td>
<td>0.772</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.091</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.016</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.689</td>
<td></td>
</tr>
<tr>
<td>2H</td>
<td>Yes</td>
<td>Two-sample t-test</td>
<td>0.621</td>
<td>0.075</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.391</td>
<td>0.129</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;0.005</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.418</td>
<td>0.120</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.027</td>
<td>0.645</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.003</td>
<td>0.947</td>
</tr>
<tr>
<td>2I</td>
<td>Yes</td>
<td>Two-sample t-test</td>
<td>0.021</td>
<td>0.658</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.012</td>
<td>0.779</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.083</td>
<td>0.414</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.012</td>
<td>0.778</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.009</td>
<td>0.815</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.143</td>
<td>0.303</td>
</tr>
<tr>
<td>3D</td>
<td>No</td>
<td>Two-sample t-test</td>
<td>0.06</td>
<td>0.433</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.06</td>
<td>0.403</td>
</tr>
<tr>
<td>3E</td>
<td>No</td>
<td>Two-sample t-test</td>
<td>0.01</td>
<td>0.928</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.14</td>
<td>0.348</td>
</tr>
<tr>
<td>3F</td>
<td>No</td>
<td>Two-sample t-test</td>
<td>0.20</td>
<td>0.239</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.10</td>
<td>0.355</td>
</tr>
<tr>
<td>3G</td>
<td>Yes</td>
<td>One-Way ANOVA with Bonferroni post-hoc</td>
<td>0.004</td>
<td>0.0917</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>3I</td>
<td>No</td>
<td>Two-sample t-test</td>
<td>0.07</td>
<td>0.199</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.02</td>
<td>0.466</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.03</td>
<td>0.238</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;0.005</td>
<td>0.363</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;0.005</td>
<td>0.482</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>3J</td>
<td>No</td>
<td>Two-sample t-test</td>
<td>&lt;0.005</td>
<td>0.509</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;0.005</td>
<td>0.630</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;0.005</td>
<td>0.552</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.002</td>
<td>0.457</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&gt;0.05</td>
<td>0.145</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&gt;0.05</td>
<td>0.086</td>
</tr>
<tr>
<td>3K</td>
<td>Yes</td>
<td>Two-sample t-test</td>
<td>0.15</td>
<td>0.290</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.45</td>
<td>0.113</td>
</tr>
<tr>
<td>3L</td>
<td>Yes</td>
<td>Two-sample t-test</td>
<td>0.30</td>
<td>0.174</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.01</td>
<td>0.683</td>
</tr>
<tr>
<td>3M</td>
<td>Yes</td>
<td>Two-sample t-test</td>
<td>0.07</td>
<td>0.447</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&gt;0.05</td>
<td>0.380</td>
</tr>
<tr>
<td>3-2H</td>
<td>Yes</td>
<td>Two-sample t-test</td>
<td>0.50</td>
<td>0.47</td>
</tr>
<tr>
<td>4A</td>
<td>Yes</td>
<td>Two-sample t-test</td>
<td>&lt;0.005</td>
<td>0.999</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;0.005</td>
<td>1</td>
</tr>
<tr>
<td>4B</td>
<td>Yes</td>
<td>Two-sample t-test</td>
<td>0.96</td>
<td>0.050</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.13</td>
<td>0.321</td>
</tr>
<tr>
<td>4D</td>
<td>Yes</td>
<td>Two-sample t-test</td>
<td>0.92</td>
<td>0.051</td>
</tr>
<tr>
<td>4-2A</td>
<td>Yes</td>
<td>One-Way ANOVA with post-hoc Tukey</td>
<td>0.602</td>
<td>0.346</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.606</td>
<td></td>
</tr>
<tr>
<td>6A</td>
<td>Yes</td>
<td>Two-sample t-test</td>
<td>1.00</td>
<td>0.050</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.02</td>
<td>0.640</td>
</tr>
<tr>
<td>7B</td>
<td>Yes</td>
<td>One-Way ANOVA with Bonferroni post-hoc</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;0.005</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;0.005</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;0.005</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;0.005</td>
<td></td>
</tr>
</tbody>
</table>

48
Table 2: Table representing the transport parameters and the turnover of mitochondria at stationary mitochondria from L4 animals anesthetized using the microfluidic device and 3 mM levamisole.

<table>
<thead>
<tr>
<th></th>
<th>Device</th>
<th>3 mM levamisole</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Init PLM</td>
<td>Mid PLM</td>
</tr>
<tr>
<td>Anterogradely moving cross</td>
<td>83%</td>
<td>66%</td>
</tr>
<tr>
<td>Retrogradely moving cross</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Anterogradely moving</td>
<td>17%</td>
<td>17%</td>
</tr>
<tr>
<td>contributes to recovery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retrogradely moving</td>
<td>0%</td>
<td>17%</td>
</tr>
<tr>
<td>contributes to recovery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total movies/total events</td>
<td>8/6</td>
<td>12/6</td>
</tr>
<tr>
<td>analyzed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterograde velocity (Dev)</td>
<td>0.40 ± 0.042</td>
<td>(n = 47)</td>
</tr>
<tr>
<td></td>
<td>0.55 ± 0.098</td>
<td>(n = 15)</td>
</tr>
</tbody>
</table>
Movie Legends

Movie 1. Time-lapse movie showing a cross event across a bleached stationary mitochondrion indicated by the arrow.

Movie 2. Time-lapse movie showing a pause at the location of a bleached stationary mitochondrion, indicated by the arrow.

Movie 3. Time-lapse movie showing a split event at the location of a bleached stationary mitochondrion, indicated by the arrow.
Extended Figures

Figure 3-1: Effect of photobleaching and intermitochondrial distances during long-term mitochondrial imaging. A, Schematic of the TRN neuron. The inset shows a single mitochondrion and a 5×5 μm box to calculate the average intensity values for a mitochondrion (M) and the background (B). B, Image of a single neuron at zero time shows a single mitochondrion (M) and the background box (B) on the worm body. The scale bar is 10 μm. C, Fluorescence ratio of the mitochondria intensity to background intensity (M/B) calculated from the images of the same animal captured at 0th, 6th, and 12th hr imaging time points. The animals were immobilized at two different time intervals of every 1 hr and every 6 hr, respectively. Time-lapse imaging of the same animal over 12 hr shows statistically insignificant photobleaching of mitochondrial fluorescence (n = 4 animals, 5 mitochondria, and a box drawn around each mitochondrion was used to calculate the statistics). Data represented as mean ± SD. Statistical significance was evaluated by one-way ANOVA with Bonferroni post-hoc comparisons; nonsignificant values (p-value >0.05) are not indicated. D, Number of events for the percentage of compression or expansion measured from relative intermitochondrial distances (ΔL/L, n = 292 events). The inset shows a pair of stationary mitochondria (M₁ and M₂) with an intermitochondrial distance of L compressed to L-ΔL. E, Average compression (M₁ and M₂ appear closer) or expansion (M₁ and M₂ move further apart) percentage values. The data represented as mean ± SEM (n = 8 animals imaged for 3 successive time points at 5 min intervals).

Figure 3-2: Kymographs of levamisole immobilized L4 C. elegans that show bleaching of stationary mitochondria and anterogradely moving mitochondria across the bleached
mitochondria. **A**, Kymograph of stationary mitochondria (red up arrow) that was bleached at 41.0 s (horizontal blue arrow) in the PLM neuronal process. The horizontal and vertical scale bars are 20 μm and 33 ms, respectively. **B**, The box and whisker plot shows the percentage drop in the integrated mitochondrial fluorescence intensity during photobleaching of n = 20 stationary mitochondria. The plot shows the median line, mean marker (×), and the outlier (o). **C**, The fluorescence intensity profile of stationary mitochondria that was bleached using a 405 nm laser. The arrow indicates the time when each of the three mitochondria were bleached at 29.0, 37.0, and 41.0 seconds. **D-F**, Anterogradely moving mitochondria (red down arrow) crosses (**D**), pauses (**E**), and partially splits (**F**) at the site of the bleached mitochondria (red up arrow). The blue horizontal line indicates the bleaching of the stationary mitochondrion. The scale bar is 10 μm. **G**, The intensity distributions along the neuronal process length and at five different time points (T₀, T₁, T₂, T₃, and T₄), represented by the red dotted lines in (**F**). **H**, The average flux of moving mitochondria across a bleached (n=15) and an unbleached (n=18) mitochondrion. The data represented as mean ± SEM. Statistical significance was evaluated by paired sample t-test; nonsignificant values (ns, p-value >0.05) are represented.

**Figure 4-1**: Time-lapse images of the neuron from an individual animal. **A**, The images of the entire neuronal process are shown at 24 and 27 hr time points after start of imaging. Two images are aligned with respect to the cell body (CB) and the two additional reference points - the branch point (BP, blue triangle) and neuronal end (Tip, red triangle) are marked on the images. The CB, BP, Tip are connected with gray dotted arrows. The trace below the images represents the neuronal processes for both time points with the mitochondria indicated as green dots. The position of fiduciary markers (CB, BP, and neuronal Tip) and three mitochondria.
are indicated at time \( t = 24 \text{ hr} \) (L₁, L₂, and L₃) and \( t = 27 \text{ hr} \) (L₁', L₂', and L₃') and connected using gray dotted arrows. The ratio of the two distances between given mitochondria from the cell body at 24 hr and 27 hr corresponds to the observed 10% (46.6 \( \mu \text{m} \)) increase in total neuronal process growth (\( \Delta L = 46.6 \mu \text{m} \)) in this 3 hr period over the initial process length of \( L₀ = 443.8 \mu \text{m} \) at \( t = 24 \text{ hr} \). B, Alignment of the neuronal process image of figure 4(f) for all 11 time points at the tip. C, A representative bubble plot of all 11 time points with the diameter of the bubble proportionate to the total area of each mitochondrion. The highlighted region shows the addition of a new smaller mitochondrion (black arrow and black triangles) between two identified adjacent mitochondria. The small mitochondrion added on 18\( ^{th} \)-hr (black arrow) increases in size in successive imaging frames. A small mitochondrion added at the 24\( ^{th} \)-hr (black inverted triangle) did not persist over 6 hr and was not considered as a mitochondrion addition event.

**Figure 4-2:** Mitochondria addition events and intermitochondrial distances measured along the neuronal processes. A, The normalized number of mitochondria added along each segment of the neuron where each segment corresponds to an equal third of the neuronal process. The total number of mitochondria addition events are 85 (9, 10, 10, 10, 11, 15, 14, and 6 for each of the eight animals imaged). Data represented as mean ± SEM. Data were compared using one-way ANOVA with post-hoc Tukey correction for multiple comparisons (ns indicates nonsignificant for \( p \)-value >0.05). B, Normalized number of intermitochondrial distances in L4 stage animals (n = 8 animals) immobilized in the microfluidic chip. The schematic of the mechanosensory neuron with cell body (CB), branch point (BP), and neuronal end (Tip). The inset shows a pair of mitochondria (M₁ and M₂) with an intermitochondrial distance \( L \).
Figure 4-3: Schematic representation of mitochondria addition events between pre-existing adjacent mitochondria in a growing neuronal process and the corresponding changes in inter-mitochondrial intervals after mitochondria additions. No new mitochondria added (A), new mitochondria added near the cell body (B), new mitochondria added at the end of the neuron (C), and new mitochondria added uniformly along the neuronal process (D). The addition of a new mitochondrion occurs when the intermitochondrial distance between adjacent mitochondria increases beyond a threshold. Three different time points are denoted that are sequentially \( t=0, t=T_1, \) and \( t=T_2 \). The red arrow points to newly added mitochondria. The dashed arrows are added to highlight the position of each stationary mitochondrion in successive time points. The panel on the right is a schematic representation of the expected change in intermitochondrial distances for each possible addition paradigm.

Figure 6-1: Intermitochondrial distance statistics when new mitochondria are added between a pair of adjacent mitochondria. A, Schematic of a mechanosensory neuron with the inset highlighting an adjacent mitochondrial pair along the neuronal process. B, Schematic of the neuronal process that shows the increase in the intermitochondrial distance between the pair of mitochondria at three different time points \( (t_0, t_0 + 3\text{hr}, \text{ and } t_0 + 6\text{hr}) \). A new mitochondrion is added at \( t_0 + 6\text{hr} \). The intermitochondrial distance is measured between the old (Pair#1) and new adjacent pairs (Pair#1A and 1B) of mitochondria at time points before and after the addition of the new mitochondrion, respectively. C, Box and whisker plot of all the intermitochondrial distances from time-lapse imaging of the neuronal processes of animals \( (n = 8) \) grown inside the microfluidic chip and imaged for 36 hr. The box represents 25% and 75% percentiles, whiskers represent outliers, and the center small box represents the mean \( (n = 85 \).
mitochondrial pairs). **D**, The bar graph of intermitochondrial distances between new pairs of adjacent mitochondria after the addition of a new mitochondrion between a pair of old adjacent mitochondria (n = 85 new pairs). **E**, The intermitochondrial distance for all 85 new pairs (Pair#1A and 1B) are plotted as a function of the normalized neuronal process location, where an addition event occurred.


Figure 1

A

B

C

D

E

F

\[ y = 0.049x + 0.079 \]

\[ R^2 = 0.85 \]

\( \text{mitochondria number} \)

Neuronal process length (µm)

Stage of the animal

\( \text{mitochondria} / 100 \mu \text{m process length} \)

% of mitochondria of a given size

Mitochondria size (µm²)

L1

L2

L3

L4

1D adult

\( \text{L1} \)

\( \text{L4} \)

\( \text{1D adult} \)
Figure 4
Figure 5
Figure 6

A

Normalized frequency

Intermitochondrial distance (μm)

0 hr 0.51 0.04
24 hr 0.52 0.0001

Data

μ²

P-value

0 hr

24 hr 1D adult

B

Normalized frequency

Intermitochondrial distance after which a mitochondria is added (μm)

P_s

P_{30 μm}

P_{51 μm}

C

Imaging time (hr)

110μm (100%)
58μm (99%)
68μm (100%)
77μm (100%)
24μm 33μm 39μm 71μm 7μm 31μm 55μm (100%)
(1%)(52%)(65%)(100%)(0%)(40%)(98%)

0-100% Probability of new mitochondria
0-110μm Intermittochondrial distance
Experimental
Prediction
Figure 7