Electronic Supplementary Material (ESI) for Lab on a Chip

Durable spatiotemporal surveillance of *Caenorhabditis elegans* response to environmental cues

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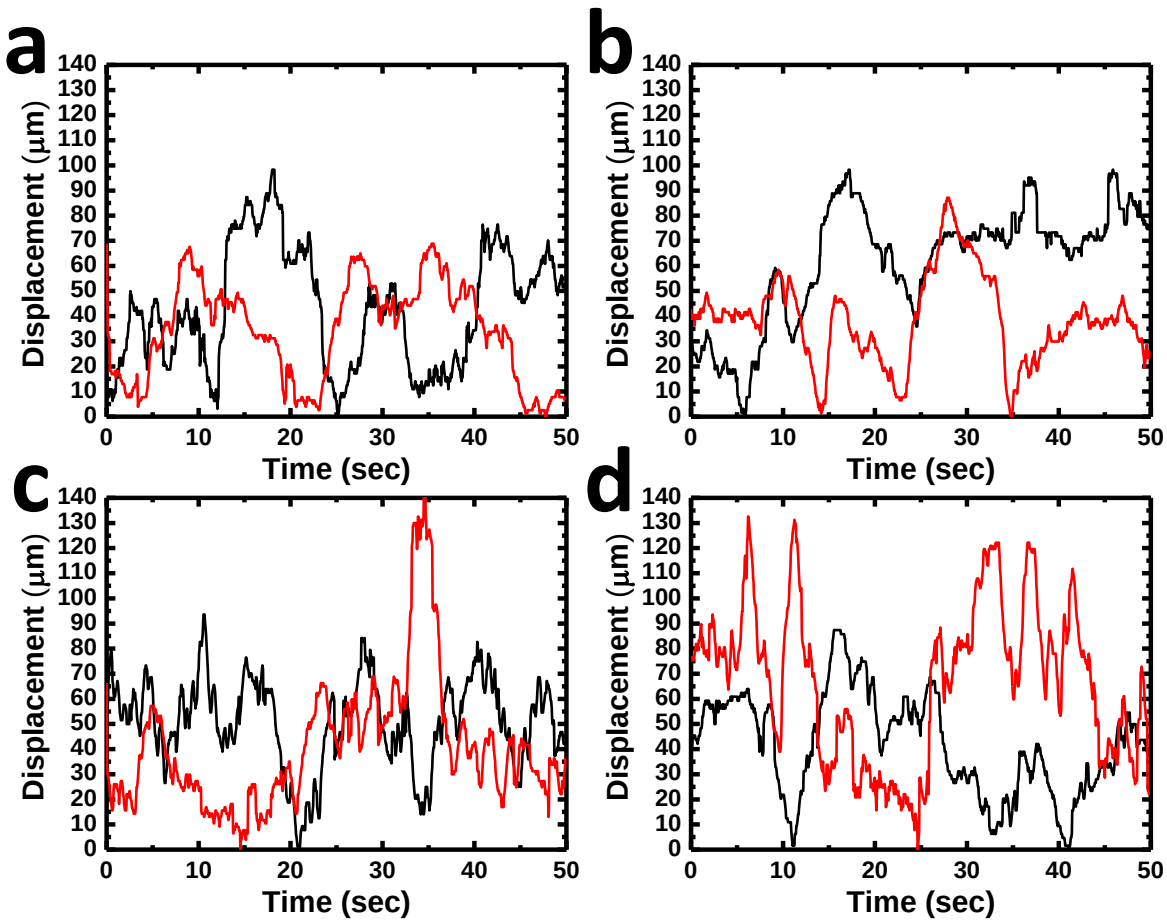
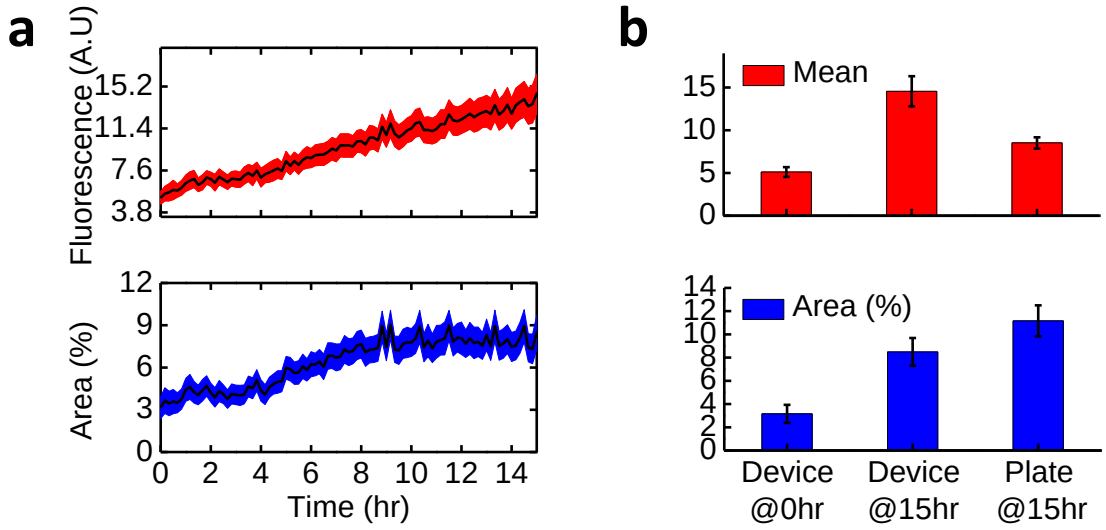
Supplementary Figures S1-S8

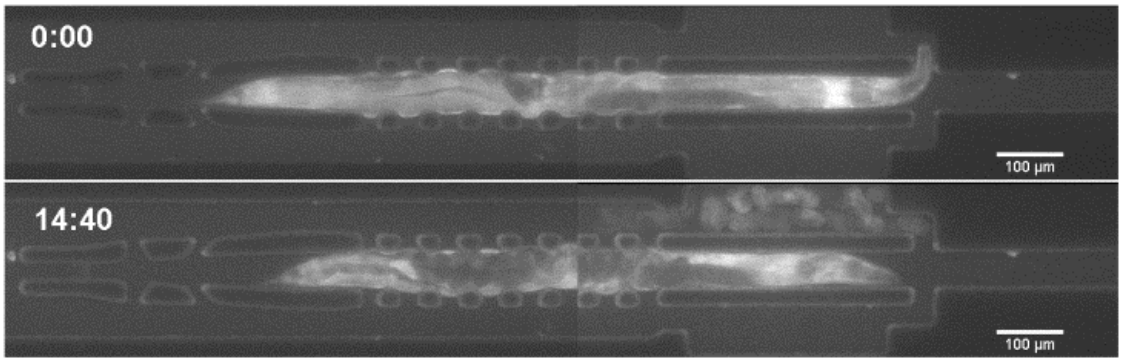
Supporting Text



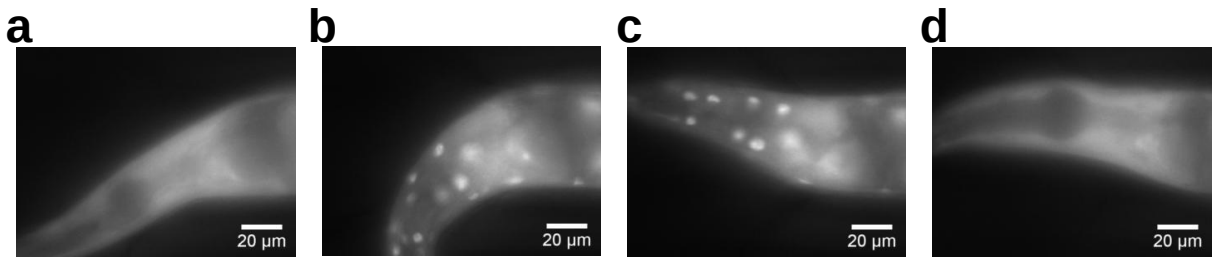
Supplementary Figure S1: Local motion of individual worms in WormSpa. Individual worms were imaged at a frame rate of 20Hz for 50 seconds immediately after loading (black) and 24 hours later (red). The position of the pharyngeal valve relative to an arbitrary fixed point was tracked automatically (see also Movie. 1). Each panel depicts the local motion of an individual worm.



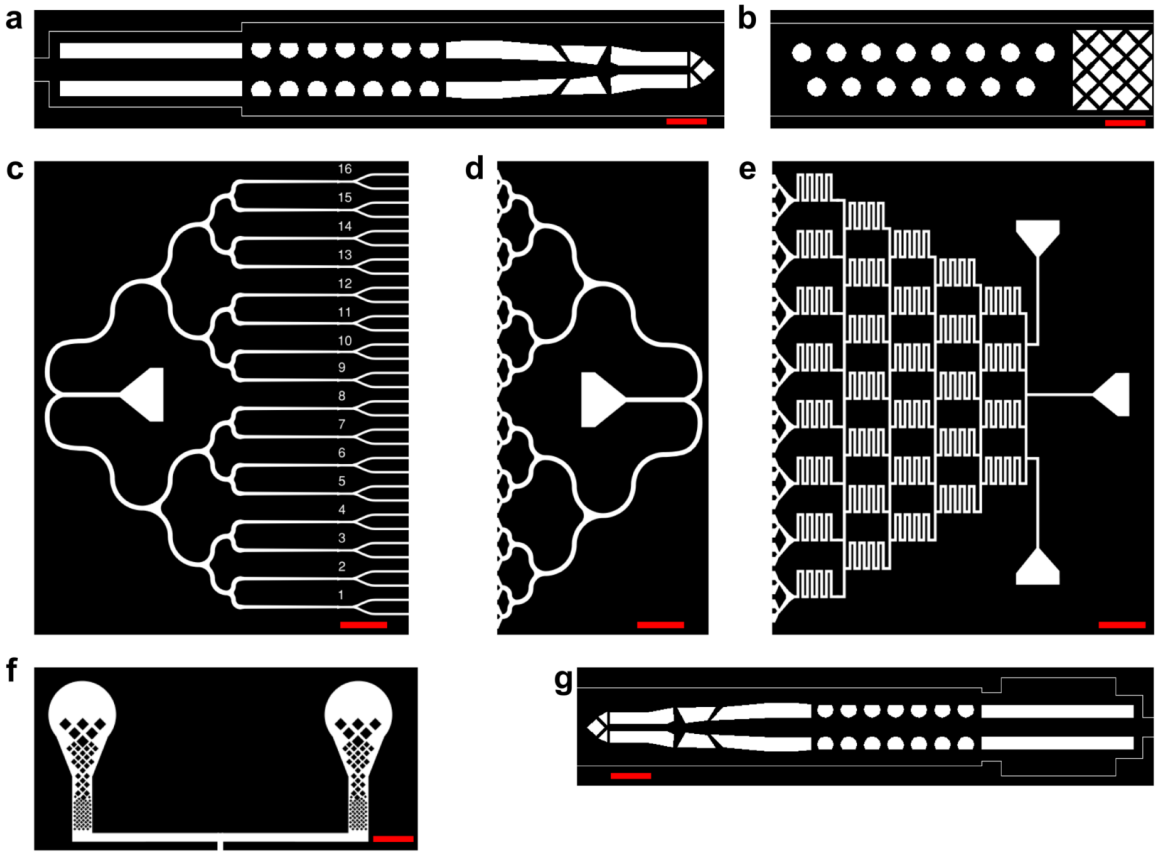
Supplementary Figure S2: Vital signs are preserved under normal conditions.
(a) Mean auto-fluorescence and the fraction of auto-fluorescent pixels (shown in red and blue respectively) for n=30 wildtype worms. **(b)** Auto-fluorescence in a population of worms (n=30) in the device at the beginning of the experiment (device @ 0hr), after 15hr (device @15hr), and in a control worm population (n=30) which remained on *E. coli* OP50 plate for 15hr.



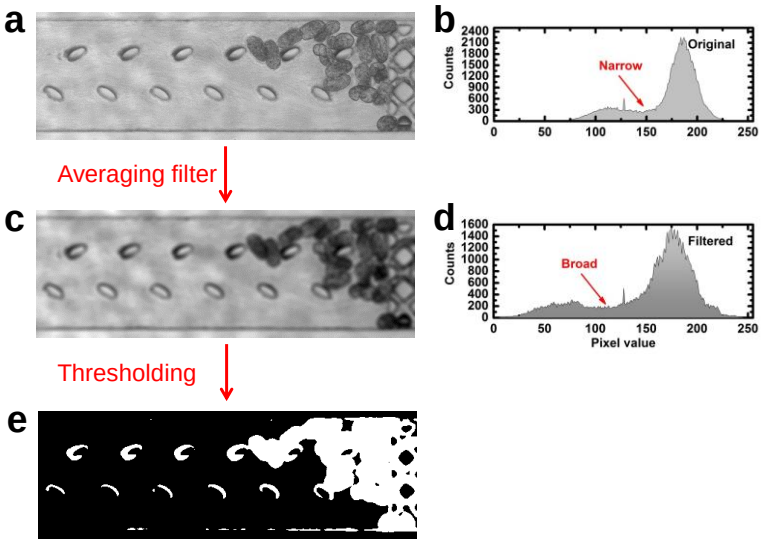
Supplementary Figure S3: DAF-16:GFP is not localized in well-fed worms.
DAF-16 remain diffusive in WormSpa as long as food is supplied, here shown a single DAF-16::GFP worm shortly after loading (**top**), and 14:40 hours later (**bottom**). This worm is shown in Supporting Movie 2.



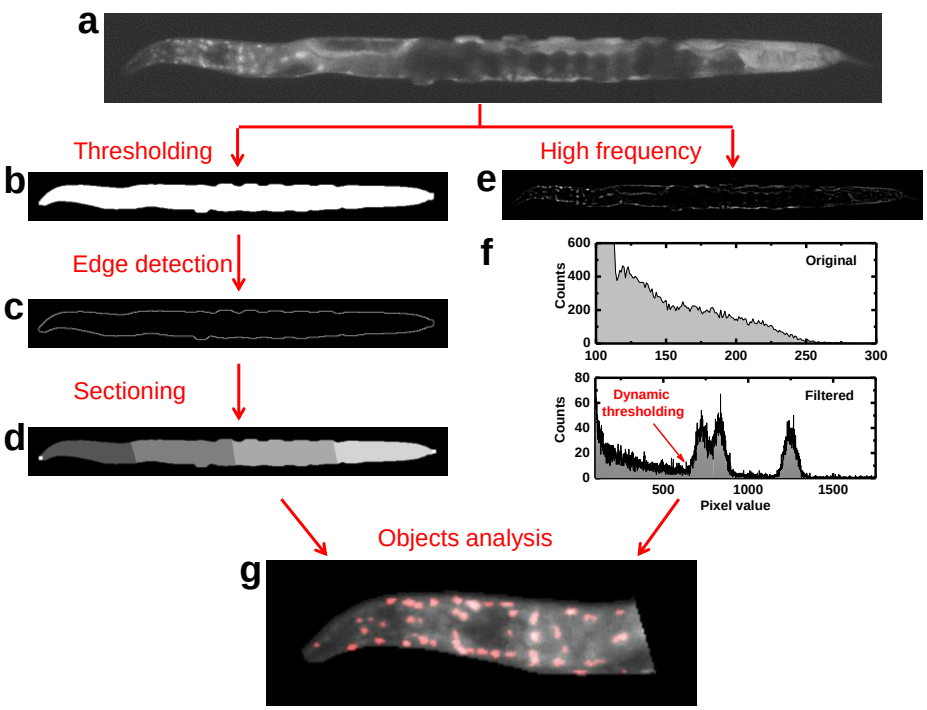
Supplementary Figure S4: Imaging Daf-16 localization at high magnification (63X). The worm head is imaged during feeding **(a)**, food depravation **(b,c)**, and 15 minutes after re-supplying food **(d)**. Images in panels **(b)** and **(c)** were taken 30 and 31 minutes (respectively) after removing food from the device. Each one of these images captures a different subset of nuclei, due to the free motion of the worm head relative to the focal plane, and the low depth of field of the objective.



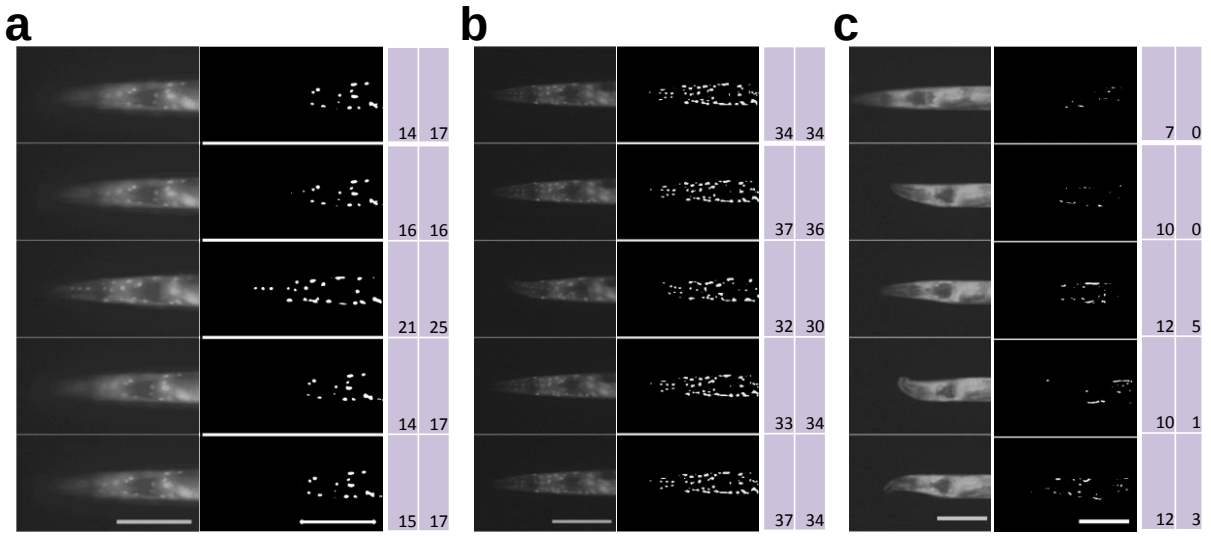
Supplementary Figure S5: Schematics of the different WormSpa modules. Scale bars are 1mm unless indicated otherwise. **(a)** Chamber (scale-bar, 100μm). **(b)** Egg-collection area (scale-bar, 100μm). **(c)** Worm-distributing channels. **(d)** Outflow channels. **(e)** Gradient module. **(f)** Step module. **(g)** Chamber with modified egg-collecting area for use with a gradient module (scale-bar, 100μm).



Supplementary Figure S6: Image analysis for egg counting. The steps of the analysis are described in the **Supproting Text.**



Supplementary Figure S7: Nuclei detection. The steps of the analysis are described in the **Supporting text**.



Supplementary Figure S8: Results of nuclei detection procedure at different magnifications. Images are from a single worm, taken within 30 seconds. **(a)** unfed worm, 20X magnification. **(b)** unfed worm, 10X magnification. **(c)** well-fed worm, 10X magnification. Next to each image is the segmented image (obtained as described in the supplementary text), the number of nuclei estimated automatically and the one counted manually. At 10X magnification cells are captured across the worm, and are therefore their number robust. The analysis basal noise level (~10 nuclei) can be estimated from **(c)**.

Supporting Text

Design of microfluidic device

Photo masks were prepared with AutoCAD, and printed at 20,000 dpi resolution (CAD/Art Services). Devices are designed as monolayer with a uniform thickness of 50 μm. The dimension of a 32 channel device is ~ 1.5cm X1 cm, and 64 channel device is ~ 2.2cmX 2cm. The basic design is composed of four modules: (1) Worm loading and distribution channels (2) Worm chambers (3) egg-collection area (4) Outflow channels. Modules 1 and 2 are common to all types of the device.

Module (1): The worm distribution channels module (supplementary Fig. S5c) is a tree-shape network, with hierarchical bifurcated branches (2^N points) that delivers the worms from the inlet to the chambers. Up to the (N-1) bifurcation point the channel width is 100μm. The channels are then straight for 3mm with a width that gradually narrows down from 75μm to 50μm. This design ensures that the total driving pressure across the device is uniform, and that the worms will be evenly distributed across it. Once a worm enters to the last bifurcation branch, the flow in the channel is blocked (resistivity become infinite), so that incoming worms are prevented from entering it.

Module (2): Worm chambers. The shape and size of the chambers were optimized to the anatomy of worms (supplementary Fig. S5a). The size of the chamber is ~ 1800 μm X 200 μm. The worm is stationed between two rows of pillars, separated by 57 μm to 25 μm. The pillars constrain the sinusoidal movement of the worm, permitting only gentle movement. They also allow the flux of food or stimuli to reach the worm, irrespective of the orientation of the worm within the chamber. Laid eggs are able to cross the row of pillars, and are flushed to the egg-collection area.

Module (3): Egg-collection area. This area (1000 μm X 200 μm) is design to accommodate the laid eggs (supplementary Fig. S5b). Two lines of Pillars are spread across it. This serves as a hinged support for the device structure. It also helps to separate eggs from each other and to ease the image analysis. The egg-collection area ends with a filter which prevents particle bigger than 10 μm to pass through it. Thus eggs remain in the collecting area, while hatched larvae are flushed out.

Module (4): Outflow channels. This module is similar to the distribution module, up to the (N-1) bifurcation point (supplementary Fig. S5d).

Optional Modules

Step module. This module is added upstream of Module (1), and includes 2 additional inlets, for buffer and substrate (or two alternative media; supplementary Fig. S5f). These inlets converge to a single channel and then to the distribution channels (Module 1).

Gradient module. Here the outflow channels module (Module 5) is replaced with a pyramidal mixing network with three inlets (supplementary Fig. S5e). This network is

capable to split, combine and mix solutions and to establish a linear concentration gradient profile as well as other complex shapes (ref. 20 of the main text). Additionally, the egg-collection area is re-designed and shifted as depicted in supplementary Fig. S5g.

Operating the device

The operation of the device is always a three steps procedure: device preparation, worms loading, and experimental mode.

Device preparation

The device is filled with 5 wt% Pluronic F127 solution (Sigma) through the outlet port. The polymer solution acts as a surfactant, it adsorbs to the surface rendering it resistant to protein or bacterial adhesion. After 15 min the device is flushed with S-medium buffer. Tubes (0.034" I.D 0.052" O.D scientific commodities) are then connected to the device in accordance with the experiment requirements. It is recommended to perform a leakage test before connecting the tubes to the syringes and to the pumps. High flow rate (>300 μl/min) of S-medium buffer for few minutes ensures that channels are bubble-free.

"Food"

Unless stated otherwise, we prepare "food" as follows. *E. coli* OP50 is grown overnight in LB media. The overnight culture (OD_{600}=3-4) is washed in S-medium, and concentrated such that the final concentration is 10 times higher than an equivalent culture at OD_{600}=1. Food can be stored at 4C for a few days.

Worms loading

Synchronized (54-58 worms are transferred from *E. coli* OP50 plate either by picking or a pipette into a micro-centrifuge tube (0.5 ml low adhesion, USA Scientific). The tube should contain in advance 100ul of "food". Worms are drawn into a loading tube using a 10 ml syringe already filled with S-medium *E. coli* OP50 mixture. The syringe tube is reconnected to a valve. Worms are injected first using the syringe pump for about 10 min (flow rate ~ 150-200 μl/min). At this stage usually most worms are perfectly aligned in the chamber. Misplaced worms can be then re-aligned manually. In our hands, 75-85% of the channels were populated by single worms with no extra effort. Rarely some of the channels were occupied by multiple worms, and withdrawn from imaging.

Experimental mode

Regular device – A reservoir containing ~15ml "food" is placed on a shaker (200rpm). The worm loading tube is unplugged from the syringe-pump, and immersed in the food reservoir. The outlet tube is connected to a syringe pump that draws liquid from the device (that is, the flow direction does not change). Flow of food was sustained at rate of 3 μl/min, short pulses of 150 μl/min were applied periodically (every 20 min).

Step device – Two input fluids are connected to the input inlets. Each of the input inlets is controlled via an independent syringe pump. For a temporal step concentration profile, we operate the inlets in a time-sequential modulation, where at any instant only

one of the input fluids is pulsed on. When the operation of the syringe pumps is swap, the exchange between mediums occurs within few seconds. For a spatial step concentration profile, both stimuli inlets are operated simultaneously. The laminar flow resists mixing between the two mediums, so that the final concentration profile has a sigmoidal shape, where the sigmoidal sharpness depends on the flow rate.

Gradient device – For a linear concentration gradient profile, solutions contain 0%, 50%, and 100% of the stimulus substrate for the Buffer, Aux, and Substrate inlet, resp. During the worm-loading step one of the inlets (usually the Substrate) serves as the outlet. Once loading of the worms is completed, the operational direction of the syringe pump is reversed and the mixing network feeds the input channels. Total flow rate is sustained at 5 µl/min, and short pulses of 150 µl/min are applied periodically.

Assays

Worm synchronization

For synchronizing worms, 20-25 adult worms were transferred to a fresh NGM plate seeded with *E. coli* OP50, and were permitted to lay eggs. Two hours later worms were removed from the plate, and the plate was transferred to an incubator at 25°C. During these 2 hours, each worm laid about 10 eggs.

Egg laying

Synchronized N2 worms (54-58hr after hatching at 25°C) were loaded into a regular device (usually 32 channels), and incubated in the chambers for 24hr with a constant supply of food (rate of 3 µl/min with periodic short pulses of 150 µl/min). Images were captured at rate of 12 frame every hour. We used phase contrast illumination (PH1 ring, N.A aperture 0.55) with ~ 30 ms exposure time, and 2X2 camera binning. Three images were necessary to cover each chamber. Data analysis was processed automatically using custom MATLAB scripts (see below).

Pumping rate

Synchronized N2 worms (54-58hr after hatching at 25°C) were loaded into regular device (usually 32 channels), and incubated in the chambers for 24hr with a constant supply of food (rate of 3 µl/min with periodic short pulses of 150 µl/min). The pharynx region was imaged at a frame rate of 35 fps for 30 sec (1050 images in total per worm) with phase contrast illumination (PH3, N.A aperture 0.55). Data were collected approximately one hour after loading, and again 24 hours later. Pumping rate was counted manually.

Survival assay

Synchronized N2 worms (54-58hr after hatching at 25°C) were loaded into a regular device (usually 32 channels), and incubated in the chambers for 24hr with a constant supply of food (rate of 3 µl/min with periodic short pulses of 150 µl/min). The worms were recovered from the device using a backflow. This was achieved by manually

applying a moderate pressure to the outlet syringe. This procedure allowed us to rescue usually more than 50% of the worms onto fresh NGM plates seeded with *E. coli* OP50. Most of the other worms remained stuck in the device, and a few got entangled in the pillars of the chamber and were damaged during the extraction attempts. During the next three days we transferred the worms to fresh *E. coli* OP50 plate each day in order to separate the worms from their progeny. We monitored survival daily by prodding each worm with a platinum wire and observing whether or not the worm moved in response to the prod. Worms that did not move were scored as dead (and were removed from the plate). As a control, in parallel to loading worms into the device we transferred other worms of the same batch to a fresh *E. coli* OP50 plate for 24hr. In the following days we applied the same counting procedure.

Food deprivation pulses

The step device was first prepared as described above. The two input fluids were sterile S-medium and "food". Syringe pump with both fluids were placed on a shaker (400 rpm). Approximately ~32 DAF-16::GFP worms (54-58hr after hatching at 25°C) were transferred from a plate to the loading tube, and were injected into the chambers. Food was first provided continuously for two hours. We then started alternating the pump actuation periodically. The flow rate was kept at 3 µl/min with rare short pulses of 150 µl/min. Time-lapse images in both GFP and PH1 channels were taken at rate of 1 frame every 5 minutes for 18 hours.

Data analysis

Egg counting

Automated image analysis procedure for counting eggs was written in MATLAB. Segmentation of eggs was accomplished through the following steps. First, the egg-collection area was chosen as the region of interest (supplementary Fig. S6a). Pixel intensity histogram was found to consist of two intersecting modes (approximately bell-shaped, supplementary Fig. S6b). The pixel values of the eggs were within the low-intensity mode (eggs appeared to be darker than the background). This suggested to us that eggs could be segmented by thresholding the image at the value of the intersection between modes. However, given the proximity of the two modes we were concerned that such a naïve approach would be too sensitive to the choice of threshold value. We therefore started by smoothing the image with an average filter (3X3), thus broadening separating the two modes (supplementary Fig. S6c and d). The resulting binary image was then inversed, and the number of white pixels was counted. For accuracy, we had to make sure that the region of interest excludes any part of the pillars or device edges. This was done using images of the device taken before eggs were laid as the baseline (supplementary Fig. S6e). We estimated that the area of a single egg in our images was 580 pixels, and therefore estimated the number of eggs as the number of white pixels divided by 580. We stress that the topology of the egg collecting area in the device keeps eggs at a single layer.

Nuclei detection and counting

Segmentation of nuclei was accomplished through the following steps:

1. Detection of worm body by thresholding (supplementary Fig. S7b). The worm body was segmented from the image background by applying an average filter (3X3), and then creating a binary image using an automatically-determined threshold. Due to the high fluorescence level of DAF-16:GFP compared to the background, thresholding is robust and insensitive to the chosen parameters.
2. Contour of the worm (supplementary Fig. S7c) – the binary image was then processed with canny edge detector algorithm. A crucial criterion for the contour is that each pixel on the contour will have only two nearest neighbors.
3. Worm endpoints (supplementary Fig. S7d) – The endpoint of the worm (anterior and posterior ends) were identified as the points of highest curvature that are separated by about half the contour length.
4. Worm sectioning (supplementary Fig. S7d) – The number N of desired sections was determined. The contour was divided into two parts (dorsal and ventral), and each part is segmented to N parts of equal lengths. Segments on opposite contours were connected, creating closed areas. For the DAF-16::GFP analysis we used N=4, and focused on the anterior-most segment.
5. High frequency filter and dynamic thresholding (supplementary Fig. S7e). Original image was convoluted with a mean filter, followed by 7X7 high frequency filter. Consequently, the values of clustered pixels (objects) enhanced greatly. The image histogram then exhibited 3 bell-shaped modes above background (supplementary Fig. S7f). Thersholding is done automatically, and only the pixels within the 3 bell-shaped modes are segmented This procedure is sufficient to discriminate between DAF-16::GFP localized nuclei and the cytoplasmic background.
6. Objects analysis (supplementary Fig. S7g) – We identified nuclei of high DAF-16::GFP concentration as connected components in the binary image of step (5) that reside within the region of interest, defined in step (4). Objects with an area bigger than 150 pixels or smaller than 5 pixels were excluded, as well as objects exceeding 20 pixels in length and objects with aspect ratio smaller than 0.5.
7. We compared the automated procedure and manual nuclei counting (shown in the tables of supplementary Fig. S8). In unfed worms the automatic procedure acts well to identify foci of expression, In well-fed worms, where Daf-16::GFP is diffusive, the false-positive rate is about 10 nuclei/worm (basal noise).

As described in the main text, images were captured using 10X objective with N.A of 0.25. Although this magnification and N.A is not commonly used for single-cell imaging, it carries some important advantages. First, the wide field of view of the objective decreases the number of images taken per worm. This increases the maximal rate of acquisition from multiple worms, and reduces the required storage space. Most importantly, the depth of field (D.O.F) of the objective is greater in one order magnitude or more compared with an objective with a higher magnification and/or N.A. For

example, the objective used in this study has a theoretical D.O.F ~10μm, whereas a 20X objective with 0.75 N.A has a D.O.F of 1.2μm. The use of a wider D.O.F alleviates focusing problems during the long experiment, and captures cells across the worm body (see supplementary Figs. S4 and S8).

Movie captions

Movie 1: Real time imaging (frame rate 20 Hz) of a worm in WormSpa. Time (in seconds) is indicated in the upper left corner. This frame rate allows for estimation of the pumping rate (here 235/min). One can observe and egg being laid and then washed towards the egg-collection area (23:95 sec into the movie). Red line is an automatic detection of the pharyngeal valve, used for estimation of local worm mobility.

Movie 2: Time lapse imaging of a worm inWormSpa, taken in phase contrast. Here the worm was imaged for over 15 hours at a rate of 6 frames/hour (time indicated in hours). Eggs are washed away into the egg-collection area. Detailed egg laying analysis for this worm can be found in the inset of supplementary Fig. 1a.

Movie 3: Pumping. High frequency (37 Hz) time lapse imaging of the pharyngeal area was captured using PH3 illumination. Pumping rate was then counted manually.

Movie 4: Time lapse fluorescence imaging of a DAF-16::GFP worm in WormSpa. Images were taken every 5 minutes for 4 hours following a 2 hour incubation in the device (time in hours is indicated in the upper right corner). Food was first delivered and then deprived for 90 minute (indicated by “ON” and “OFF”, respectively), resulting in enrichment and depletion of DAF-16 nuclear localization at the head region.