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A high-throughput device for size based separation of *C. elegans* developmental stages

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Abstract

Caenorhabditis elegans is a widely used model organism to study development, aging and behavior. Many of these biological studies require staging a large number of worms to assay a synchronized population of animals. Conventional synchronization techniques such as manual picking, gravity stratification and chemical bleaching are labor-intensive and could perturb animals' physiology. Thus, there is a need for a simple inexpensive technology to sort a mixed population of worms based on their developmental stages with minimal perturbation. Here we demonstrate a simple but accurate and high-throughput technique to sort based on animal size, which correlates well with developmental stages. The device consists of an array of geometrically optimized pillars that act as a sieve to allow worms of specific sizes to rapidly move through. With optimized chamber heights, pillar spacing and driving pressures, these binary separation devices are capable of independently separating a mixture of worms at two different stages at average efficiency of around 95%, and throughput of hundreds of worms per minute. In addition, when four devices are used sequentially, we demonstrate the ability to stratify a mixture of worms of all developmental stages with >85% overall efficiency.

Introduction

Caenorhabditis elegans (*C. elegans*) is a widely used model organism in genomics, neurobiology, developmental biology, and aging studies because of its well-characterized genome and developmental processes and its fully mapped neural circuitry^{1–6}. In addition,

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[†]Electronic Supplementary Information (ESI) available: Chamber ceiling heights at operational pressures superimposed on animals' diameters for each device. Five movies demonstrating sorting a mixture of two adjacent developmental stages using the optimized devices, respectively. See DOI: 10.1039/b000000x/

its transparent body, short lifespan and hermaphroditic reproduction allow ease of culture and manipulation and compatibility with live fluorescence imaging⁷. The life cycle of *C. elegans* consists of four developmental larval stages (L1–L4) and an adult stage, each of which exhibit different yet characteristic body sizes, and morphological and anatomical features. *C. elegans* can also develop into a special larval stage called the dauer diapause in order to survive under unfavorable environmental conditions⁷. Dauer animals can be morphologically distinguished from other stages by their small, thin bodies⁸.

Many biological research assays require an isolated population of worms at the same developmental stage⁹. This is conventionally accomplished by manual picking, gravity stratification, chemical synchronization via bleaching and Sodium Dodecyl Sulfate (SDS) treatment to isolate dauers⁷. However, these approaches have various drawbacks, such as time-consuming manipulation, labor-intensive operation, inaccurate and inconsistent results, and possible perturbations to worms' physiology¹⁰.

Commercially available automated sorting devices such as the COPAS Biosorter can be used to sort worms, but are expensive and may not be accessible to many labs¹¹. Microfluidics has emerged as an alternative for manipulating worms in behavior, genetics, screening as well as automated imaging and analysis. Several high-throughput worm sorters have been developed that sort according to reporter gene expression level or other fluorescent markers^{12–16}. Although automated, these systems are not designed for sorting based on age and size, and they are operationally complex. In contrast, several devices have been developed recently that sort worms based on size and other age-dependent properties. For example, there are devices that utilize electrotaxis and worm behavior within mazed arrays as driving forces for age separation^{17–20}. While electrotaxis is an interesting method of controlling worm directional movement, the precise mechanisms of electrotaxis are not fully understood and the effects on worms are unknown^{18, 19}. In addition, the existing devices have a trade-off between a sufficient throughput and accuracy of sorting. For example, while Rezai *et al.*'s device has a high throughput of 78 worms per minute, relative to that of Maniere *et al.* (four worms per minute), both devices have low sorting accuracies^{18, 19}. Han *et al.*'s recent device takes advantage of electrotaxis as well as size-dependent motility in microstructured channels which they managed to achieve an accuracy of around 95% but a low throughput of around 4.3 worms per min¹⁷. Solvas *et al.*, on the other hand, diverged from the use of electrotaxis as the principle in worm sorting, and used smart maze arrays and size differences to passively sort only adult from larvae worms²⁰. Therefore, until now, there has not been a microfluidic device capable of separating worm mixtures into individual larvae and adult stages with high throughput and accuracy and minimal perturbation to worms' physiology. Here we present a simple and robust system that meets these criteria. We demonstrate accurate separation of various stages of worms, including larvae, adults, and dauers at a rate of hundreds of animals per minute in a single device.

Methods

Design principle

The fundamental principle used for this work is size-based flow filtration, similar in principle to some cell size sorting devices and certain modes of gel electrophoretic separations of macromolecules. One potential problem of size filtration is the tendency for particulates in the filtrate to clog passages and hinder the continuous operation of the device. This challenge was addressed in our system by using an array of geometrically optimized pillars in the main chamber (60 μm diameter, Fig. 1) ²¹. The width and length of the chamber are 15 mm by 4 mm, respectively. Pillar spacing is measured as the closest edge to edge distance between pillars. These pillars are reversibly bonded to the chamber floor, acting as a filter to allow only worms of a specific small size to continuously flow through the device while trapping larger worms. However, because these pillars are reversibly bonded^{22, 23}, they can detach under elevated pressure in order to release larger worms at a later time (Fig. 1). The devices also contain a second valve control layer to control the sample collection.

To separate a mixture of worms into their corresponding 5 developmental stages, four devices were designed with varying chambers heights and pillars spacing. By changing these two geometric parameters as well as operation pressure then quantifying the speed at which the different sized worms travel through the chamber we were able to optimize individual devices' size filtration ability.

Device fabrication

The devices were fabricated using standard multi-layer soft lithography techniques.^{24, 25} Features from the main chamber and valve layers were transferred from transparent masks to silicon wafers by spin-coating SU8-2010, SU8-2025, and SU8-2050 (MicroChem) according to desired feature heights (Table 1). These wafers were then treated with tridecafluoro-(1,1,2,2-tetrahydrooctyl)-1-trichlorosilane silane (UCT Specialties, LLC). For all devices, the main and valve layers were molded with a PDMS (Sylgard 184, Dow Corning) mixture of 10:1 base polymer to cross-linker ratio, with exception of device I, where a PDMS mixture of 20:1 was used instead. The chamber layer was then spin-coated with PDMS to achieve a membrane thickness of 20 μm before both layers were partially cured at 70°C. The two layers were then aligned together and thermally bonded at 70°C overnight. To construct reversibly attached pillars, the chambers were protected by a blank PDMS stamp prior to being plasma treated (PDC-32G plasma cleaner) and bonded onto glass coverslips.

Device operation and characterization

A worm mixture of two adjacent developmental stages is loaded into each device. The device works in a semi-batch manner with a total of three different control phases: loading, flushing and releasing (Fig 1b). During the loading phase, with loading and outlet 1 valves open, pressure is applied to load the worm mixture into the chamber; larger worms are trapped between the pillars allowing only the smaller worms to pass through. As the accumulation of larger worms starts to hinder the passage of smaller worms, a flushing

phase is initiated by opening only the flushing valve which will then drive the remaining smaller worms out of the chamber. With the smaller worms out of chamber, the pillars are detached to allow larger worms to finally flow through by increasing the flow pressure to 11 psi with outlet 2 opened. An empty chamber marks the end of the sorting batch and the process is repeated until all worms are sorted to their respective outlets.

Two types of experiments were performed: characterization and sorting. Videos of both sorting and characterization experiments were recorded at an acquisition rate of 15 fps using Infinity 3 CCD camera (Electron Microscopy Sciences, Hatfield, PA). Characterization and geometry optimization of each device were performed for each binary worm stage device. For these experiments, the speed of worms traveling through the device was the only metric used to determine the device's principle filtration ability at a particular geometry. To determine average speed, we manually tracked worms from device entrance to exit and converted the numbers of elapsed frames to average worm speed. Sorting experiments were performed for each optimized device while measuring numbers of both true and false positive worms at each outlet as a metric in scoring sorting accuracy. Images of worms of all developmental stages were captured as standards for visual quantification, assuming the body morphology of larval and adult worms are consistent when grown under identical culture conditions.

Worm locomotion, pharyngeal pumping rate and body bending frequency were analysed manually using ImageJ video analysis. All statistical analyses were performed using two sampled t-tests assuming equal variance.

***C. elegans* strains, culture, and assay**

C. elegans strains used in these studies were wild-type N2, QH3736 *lon-3(e2175); zdlIs5(Pmec-4::GFP)*, QH3833 *dpy-4(e1166); julIs76(Punc-25::GFP)*, CB1611 *mec-4(e1611)*, and QL381 *daf-7(Pdaf::GFP)*. All strains were cultured on Nematode Growth Medium (NGM) plates seeded with OP50 strain of *Escherichia coli* and maintained at 20°C using established culturing protocol.⁷ To synchronize worms, embryos were obtained from gravid adult hermaphrodites by treatment of bleach solution containing 1% NaOCl and 0.1 M NaOH, allowed to hatch in M9 buffer, then cultured onto NGM plates seeded with OP50. Animals were washed and suspended in M9 buffer containing 0.01 wt% Triton X100 as a surfactant for experiments.

To determine the devices' deformation under pressure (Fig S1), all devices were filled with a solution of Albumin-fluorescein isothiocyanate conjugate (BSA-FITC, 0.2 µM, Sigma) and their cross-sections imaged using a confocal microscope (Zeiss LSM 510 VIS).

For viability experiments, L4 larvae and adult worms were exposed to the same pressure, temperature, and flow conditions in the devices as worms going through separation experiments. Control groups were kept in liquid M9 solution for the same duration. Both control and experimental groups were then placed on OP50 seeded NGM plates and their pharyngeal pumping rate and body bend frequency were compared. For the long term survival test, two L4 larvae populations underwent identical sorting experiment preparation but only one worm population (n ~ 100) were injected into device IV and experienced the

same conditions as worms of a normal sorting experiments. After the experiment, a random subset of 10 worms were collected from both the experimental and mock groups and cultured separately on OP50 seeded NGM plates for three days. This experiment was repeated three times. The numbers of eggs laid on each plate was counted daily before the worms were relocated onto new plates.

For experiments regarding the separation mechanism, a group of L4 larvae treated with 10 mM sodium azide (Sigma Aldrich) as an anaesthetic were injected and pumped through a sorting device to assess flowing speed after all worms were immobilized. A control group of L4 larvae without sodium azide treatment was also tested in an identical fashion. In addition, the speed of *lon-3*, *dpy-4*, and *mec-4* mutant young adults going through device IV at 5 psi was also quantified in a similar manner.

Results and Discussion

Parameter optimization

Two parameters were optimized in order to obtain the highest sorting accuracy and throughput: chamber height and pillar spacing. The chamber heights and pillar spacings tested with each device are listed in Table 1. The chamber was designed so that its height is between the body diameters of the two stages during the loading phase, but larger than both stages when the device is under higher operating pressure during the releasing phase (Fig. S1). Because worm sizes can still vary within a developmental stage, this feature is not the only mechanism to retain larger animals, but it does help hinder the movement of larger worms. Two main criteria were considered while optimizing device geometry: how well the pillar geometry traps the larger worms and the absolute difference in the speed of the two different staged worms through the chamber. Ideally, larger worms will be completely immobile while the smaller worms move as fast as possible through the device in order to achieve high separation efficiency and maximize throughput.

As shown in Figure 2, the difference between the speed of smaller and larger worms is a strong function of pillar spacing, device height, and operating pressure. In addition, specifically for devices I and III (Fig. 2a and 2c), at the largest pillar spacing, larger worms were not completely immobile, which can affect sorting accuracy. Therefore, we chose to maintain sorting accuracy by eliminating potential escape of larger worms through the device, slightly sacrificing sorting throughput by choosing medium pillar spacings (80 μm , 60 μm , 120 μm , and 240 μm pillar spacings for devices I, II, III, and IV, respectively).

In addition, we quantified worm speed as a function of fluid pressure, shown in Fig. 3. Increasing the fluid pressure results in smaller worms moving faster through the chamber and larger worms remain relatively immobile. The difference in speed of large and small worms is significant above a fluid pressure of 2 psi; this indicates that although sorting throughput varies, worms can be accurately filtered by size at any pressure above 2 psi.

Evaluation of separation efficiency

For each experiment, we collected two populations: worms that exited the device during the loading stage (outlet 1) and worms that exited the device during the releasing stage (outlet

2). According to our design, the smaller animals will ideally be sorted to outlet 1 while the larger animals would be sorted through outlet 2. To evaluate the efficiency of the separation process, we considered two descriptors: purity, which is defined as the number of target worms in each outlet divided by the total number of worms exiting that outlet, and efficiency, which is defined as the number of target worms in the correct outlet divided by the total number of target worms loaded (Table 2). Overall, the devices were able to achieve an average efficiency greater than 95% and an average throughput of 129 ± 31 worms/min with a maximum of 180 worms/min.

Besides separating the larvae stages, these devices were also able to separate dauer animals from the L4 stage. The dauer stage is an alternative diapause stage studied for its reduced aging rate and stress resistance, and is important in several areas of developmental biology. Dauers could be readily separated from L4 animals using a device of 25 μm chamber height and 120 μm pillar spacing at efficiencies of over 95% (Table 2). Videos of sorting operation for each device can be viewed from the supplementary materials.

Finally, we demonstrated that these devices can be used to separate a natural mixture of worms into populations of the five normal developmental stages (L1–L4 + adult). This, to our knowledge, has never been done before using a simple microfluidic device. The four optimized devices were set up in sequence as shown in Fig. 4a. A mixture of L1, L2, L3, L4 and adult worms were fed into the first device (device IV), which isolated the adults from the rest of the larvae mixture. The remaining worms were then fed into the second device (device III) to isolate L4's. This process continued with the remaining two devices (devices II and I) until worms of all five stages were isolated into their individual bins. The purity of worms at each stage after sequential separation is shown in Fig. 4b. There was a general decrease in the purity of this experiment compared to that of a single device. This is not unexpected, and most likely due to the retention of inaccurately sorted worms throughout the sequential setup. Nonetheless, the purity achieved here is adequate for most biological experimental need.

Effect of the devices on Viability

To ensure that the operation conditions of these devices do not result in physiological damage to the worms, we evaluated worm physiology immediately post experiment and long term effects. Physiology was measured with two indicators: pharyngeal pumping rate and body bending frequency post separation. Long term effects were measured by looking at percentage of survival three days post experiment and their fecundity throughout those days. There were no statistically significant differences in these parameters between sorted adults and L4 worms and their non-sorted control groups (Fig. 5). The pumping rate of ~ 250 pumps/min and bending frequency of $\sim 0.4\text{Hz}$ are consistent with reported literature values.^{21, 26} This suggests that sorting conditions have no discernible adverse effects on the physiology of the worms.

For the long term survival assay, we compared a group of worms that have undergone the sorting experiment with a control group. All worms of both populations survived after 3 days of culturing under standard conditions after actual and mock sorting experiments (data not shown). In addition, we found no statistical differences in their fecundity throughout

these three days (Fig. 6). These results show that the sorting experiments have no detrimental effect to the worms' immediate physiology post experiment and their long term survival and fecundity.

Separation Mechanism

To demonstrate that size is the major factor affecting transport speed within the chamber, we have performed two additional experiments. First, we quantified the speed of two mutant strains with growth defects through the device chamber to show that speed within the device is dependent on size and not on other age or developmental related differences. Second, we then compared the speed of mechanosensitive mutants (*mec-4*), anesthetics-immobilized worms and wild type animals to demonstrate that speed through the device is mainly due to the convective push of fluid flow and does not require coordinated worm movement.

The two different-sized mutants we tested were *lon-3* and *dpy-4* mutants: *lon-3* animals are 50% longer than wildtype,²⁷ and *dpy-4* animals are shorter and thicker than that of wild type.^{28, 29} The speed of wild type N2 animals was significantly greater than both *dpy-4* and *lon-3* animals (Fig. 7). By visually inspecting animals moving through the device, we found *lon-3* animals were more easily trapped between chamber pillars, presumably due to their longer length, therefore reducing their collective speed through the device. Similar results were observed with *dpy-4* animals. The thick body of *dpy-4* animals makes them stiffer and less agile in bending and evading the pillars as they flow through the chambers. These experiments confirmed that size is an important parameter driving separation of the worms, and is not solely dependent on their developmental stage.

mec-4 mutants lack an amiloride-sensitive sodium channel required to sense gentle mechanical stimuli along the body wall.³⁰ Their speed through the device is not statistically different from that of wildtype animals at the same developmental stage (Fig. 7). This suggests that any seemingly navigation and pillar evasion techniques used to facilitate an escape from the chamber were not a result of mechanosensation. This was further examined by quantifying the travelling speed of L4 worms immobilized by NaN₃, a chemical commonly used to reversibly anesthetize *C. elegans* by inhibiting both cytochrome c oxidase and adenosine triphosphate (ATP) synthase.^{31–33} The speed of anesthetized N2 worms moving through the device is not statistically different from that of untreated N2 worms (Fig. 7). These two experiments together demonstrate that the speed of worms within the device is mainly due to the convective push of fluid current through the device and not dependent on the worms' mechanosensation and locomotive behavior. This is a critical feature unique to this device that is advantageous when dealing with strains with various intrinsic behavioral differences. This allows separation of worms based on only their size differences; thus the device is likely useful for other strains of worms independent of behavior variability.

Conclusion

In this paper, we introduce a simple microfluidic device capable of sorting *C. elegans* based on size difference with an average efficiency of over 95% and throughput of over one hundred worms per minute. Compared with other worm sorting devices and conventional

age synchronization methods, this device requires minimal labor, is inexpensive, minimally perturbs the worm physiology and has an unprecedented accuracy and throughput. Additionally, since this device operates purely based on size instead of behavioral differences, it is capable of sorting behavioral mutant animals and dauer animals, a capability that is completely unique to this device.

Moreover, this device can be easily automated and incorporated upstream of most compatible microfluidic devices for fast and accurate preparation of age synchronized populations. This can be a powerful addition to microfluidic systems that heavily rely on accurate age or size synchronization of a large population of animals such as research related to developmental diseases and age or size specific drug screening. Its low cost, versatility and operationally simplistic nature allows it to potentially replace conventional worm sorters and synchronization methods and be universally adapted to many *C. elegans* related research facilities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Overall design and operation. (a) Device design. Under low fluid pressure, rigid pillars restrict movement of larger worms through the device. However, at higher pressures, pillars are detached from the glass substrate, allowing movement of larger worms. (b) Operating procedure for sorting two worm populations. White background indicates low chamber pressure; grey indicates high chamber pressure. Larger worms are depicted in red while smaller worms are in yellow. Open and closed valves are shown in white and pink, respectively.

Fig. 2.

Effects of various pillar spacing and device height on worms' velocity within individual devices. The fluid pressures and chamber heights used are: (a) 3 psi and 15 μ m; (b) 2 psi and 25 μ m; (c) 5 psi and 25 μ m; (d) 5 psi and 45 μ m. Values are expressed as mean $\pm$ SEM, $n = 30$ for each, * $p < 0.05$, ** $p < 0.01$.

Fig. 3.

Effect of fluid pressure on worms' velocity within individual devices. Values are expressed as the means $\pm$ SEM, $n = 30$ for each, * $p < 0.05$, ** $p < 0.01$. Optimized pillar geometries for each device were used according to Table 1.

Fig. 4. Series separation of worm mixture. (a) Schematic of four optimized devices set up in sequence for sorting a worm mixture of all developmental stages. (b) Plot of purity for sorted worms. Results are representative of 3 independent measurements. Average numbers of worms used for each experiment are: 195 ± 71 , 133 ± 34 , 230 ± 110 , 215 ± 63.5 , and 250 ± 65 worms at L1, L2, L3, L4 and adult worms, respectively. Error bars show SEM.

Fig. 5.

Analysis of worm viability post-separation. (a–b) Pharyngeal pumping rates of L4 and adult worms. (c–d) Body bend frequency (f) of L4 and adult worms. $n = 15$. Exp. and Con. represent experimental and control groups, respectively.

Fig. 6. Fecundity post device treatment. Eggs laid per worm per day for experimental and control groups throughout three days post experiment. $N = 10$ worms per measurement. Error values are expressed as $\pm$ SEM. $p > 0.15$.

Fig. 7.
Speed of L4 *dpy-4*, *lon-3* and *mec-4* mutant worms and N2 control and sodium azide treated N2 worms through Device IV. Error values are expressed as $\pm$ SEM, n = 30 for each, *p<0.05, **p<0.01.

Table 1

Various pillar array geometries and sorting conditions tested. Red dashed lines represent the parameters ultimately used.

Device	Stages of worms	Chamber height(μm)	Pillar spacing (μm)	Flow pressure (psi)
I	L1 & L2	15	30, 80, 180	1, 3, 5
II	L2 & L3	25	30, 60, 180	1, 2, 5
III	L3 & L4	25	60, 120, 240	3, 5, 8
IV	L4 & Adult	45	120, 240, 360	3, 5, 8

Table 2

Separation efficiency and outlet purity

Device Used	Target		Purity (%) ^a		Efficiency (%) ^b	
	1	2	Outlet 1:	Outlet 2:	Outlet 1:	Outlet 2:
I	L1	L2	97±2	92±4	94±2	95±3
II	L2	L3	94±3	97±1	96±3	94±2
III	L3	L4	96±2	89±11	93±6	94±4
IV	L4	Adult	99±1	98±1	99±1	97±4
III	dauer	L4	98±3	95±1	98±1	97±5

^a Purity is defined as the number of target worms divided by the number of total worms in each outlet.

^b Efficiency is the geometric average of the number of target worms in each target outlet divided by the number of total target worms loaded. Results are representative of 3 independent measurements. Each measurement of the experiment was performed with 322 ±70 worms.