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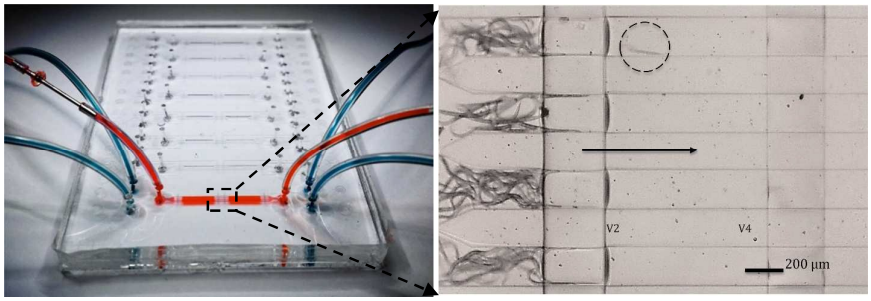
A new microfluidic approach for size-dependent sorting of C. elegans nematodes and extraction of embryos for age-synchronized worm populations.



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PAPER

Versatile size-dependent sorting of *C. elegans* nematodes and embryos using a tunable microfluidic filter structure†

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The roundworm *Caenorhabditis elegans* (*C. elegans*) is a powerful model organism for addressing fundamental biological questions related to human disease and aging. Its life cycle consists of an embryo stage, four larval stages that can be clearly distinguished in size and by different morphological features, and adulthood. Many worm-based bio-assays require stage- or age-synchronized worm populations, for example for studying life cycle and aging of worms under different pharmacological conditions, or to avoid misinterpretation of results due to overlap of stage-specific response in general. Here, we present a new microfluidic approach for size-dependent sorting of *C. elegans* nematodes on-chip. We take advantage of the external pressure-deformable profile of polydimethylsiloxane (PDMS) transfer channels that connect two on-chip worm chambers. The pressure-controlled effective cross-section of these channels creates adjustable filter structures that can be easily tuned for a specific worm sorting experiment, without changing the design parameters of the device itself. By optimizing the control pressure settings, we can extract larvae of a specific development stage from a mixed worm culture with an efficiency close to 100% and with a throughput of up to 3.5 worms per second. Our approach also allows generating mixed populations of larvae of adjacent stages, or to adjust the ratio of these directly in the microfluidic chamber. Moreover, using the same device, we demonstrated extraction of embryos from adult worm populations for subsequent culture of accurately age-synchronized nematode populations or embryo-based assays. Considering that our sorting device is merely based on geometrical parameters and operated by simple fluidic and pressure control, we believe that it has strong potential for use in advanced, automated, microfluidic *C. elegans*-based assay platforms.

Introduction

C. elegans has a fully sequenced genome of approximately 20,000 genes of which about 60-80% have human homologues. This is only one of the reasons why over the last decades *C. elegans* has become one of the most popular model organism to understand, among others, human disease and aging [1-4]. The nematode shares many conserved systemic, molecular and genetic mechanisms with humans [5-7]. Furthermore, each hermaphrodite has only 302 neurons of which the physical connections have been fully mapped out [1, 2, 8, 9]. *C. elegans* is therefore widely used for genetic studies, developmental biology, molecular biology, neurobiology, biochemistry and human disease research [10-12]. In addition to merely biological reasons, more practical features make *C. elegans* a powerful tool for these studies. First, *C. elegans* is usually simply cultured at or close to room temperature on agar plates or in liquid medium with *E. coli* strains OP50 or HT115 as food source [13]. As culture conditions are quite robust, it is easy to maintain *C. elegans* populations in any, also non-biological, laboratories. Second, even adult *C. elegans* worms are of small size (length ~1 mm, diameter ~50 µm) and have a transparent body. Various imaging techniques may be readily applied to study the animal's anatomy, inner organs, nervous systems etc., including high-resolution transmission bright-field microscopy and fluorescent imaging. Most biological assays can be directly carried out on the agar plate or in liquid medium on-chip. Third, it has a relatively short development time from embryo stage to adulthood (~3.5 days at 20 °C) and a lifespan of only 2 to 3 weeks. Each development stage, in particular the four larval stages (L1-L4), can be clearly distinguished by the worms' body size and specific morphological features.

Microfluidic chip-based assays recently emerged as a new and versatile technology for studies on *C. elegans* [14-39]. Microfluidic approaches allow accurate worm manipulation and advanced handling protocols, for instance for assaying worm populations in specific microchambers, or immobilization of single worms for high-resolution microscopic imaging without using anesthetics. As an example, Chokshi *et al.* developed a device where a worm was pushed and fixed for imaging at the side a microfluidic channel by deformation of a flexible PDMS membrane [14]. Behavioral studies and recording of neuronal activity under stimuli, like exposure to chemical compounds, light and temperature [21, 26, 34], as well as laser ablation [27] and nerve regeneration studies [28, 33] have been performed on-chip. Additionally, high-throughput imaging, phenotyping and

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screening have been successfully performed by using microfluidics technology [28-32].

C. elegans has been at the forefront of aging research since the first long-living mutant was discovered over 25 years ago [40, 41]. Studies of aging in *C. elegans* focus on the identification of factors that influence life span and the analysis of the underlying mechanisms responsible for longevity. Such longitudinal studies are performed best by using stage- and/or age-synchronized cohorts of animals. Likewise, in many other worm assays, synchronized worm populations are also advantageous in view of better targeting specific experiments and reducing the variability of results. For the time being, synchronization still frequently relies on manual selection of a large number of *C. elegans* larvae [42]. Two classical methods, which are both based on the time synchronization of embryos, are mainly used: (i) exploiting a timed embryo lay or (ii) bleaching of gravid worms to isolate embryos. For synchronization via a timed embryo lay, adult worms are allowed to lay embryos for a defined period of time, typically 2–6 h. Subsequently, after a sufficient number of embryos has been produced, all adult worms are removed from the plate [13]. Bleaching dissolves the worm body and liberates the embryos from the adult worm in the medium. However, these methods are time-consuming, labor-intensive and inaccurate since they depend strongly on the visual acuity of the operator. On the other hand, automated sorting systems, such as the fluorescence-activated COPAS Biosort platform (Union Biometrica), provide high-throughput and, for instance, enable the isolation of mutants with altered fluorescent protein expression [43]. However, such equipments are less suitable for sorting and isolation of larval stages.

Several chip-based devices have been developed recently that allow sorting, *i.e.* the extraction of a desired sub-group from a mixed larvae/adult worm population, based on size and/or behavioral differences. For example, one has utilized electrotaxis as driving force, *i.e.* the tendency of nematodes to migrate towards the negative pole of an electric field, often combined with worm behavior in maze arrays, to direct the motion of the animals [44-49]. By collecting animals at a predetermined location, one can achieve motility-based separation as a function of their arrival time. Solvas *et al.* used a smart microfluidic maze array to passively isolate adult worms from larvae on the basis of size and age [44]. Rezai *et al.* spatially separated different stages of *C. elegans* in two microchambers connected by a constricted electric trap region [45]. Han *et al.* proposed micro-bumps arrayed channels which allow size separation due to restriction of the undulating motion of the worms passing through [46]. While electrotaxis is now a well-known technique of guiding the worm through fluidic microstructures [45, 47], it is also known that long time exposure to electric fields may adversely impact the animals' motility, which limits the accuracy of sorting [45, 47]. For the time being, it is not clear to which extent the electric field experienced by the worms has an impact on subsequent bio-assays. Besides, this method suffers from a relatively low throughput and is limited to strains that exhibit electrotaxis [46]. In contrast, Ai *et al.* developed a simple sorting device based on differences in animal size and morphology [50]. The device consisted of an array of geometrically optimized pillars that acted as a filter, allowing worms of specific sizes to move through more rapidly. This technique is simple, accurate and allows high-throughput sorting of worms of different sizes. The drawback of all these devices is that they feature filtering structures with fixed geometrical dimensions. As a consequence, for successful sorting or size separation of different worm populations, such as larvae stages, male/hermaphrodite worms or different mutants, the device design and several parameters have to be adapted for each specific experiment. This obviously limits the versatility of the device. Clogging by the debris or *E. coli* clusters may also occur easily, especially when using small filters sizes for young larvae. This issue impedes continuous and long-term operation of the device.

Here, we propose a simple microfluidic chip that allows size-dependent sorting of different stages of *C. elegans* with comparatively high throughput. A single device can be used for selecting and sorting of all worm stages. The design is based on the concept of external pressure-controllable PDMS channels [51], where we take advantage of the adjustable cross-section of a microfluidic microchannel to create filter structures that can be easily tuned for a specific worm sorting experiment. Pressure settings were optimized for size-separation (*i.e.* sorting) of the different animal stages. We demonstrate the feasibility of separating mixed populations of larvae of all stages (L1 to L4) and adult worms. Our approach also allows generating populations of adjacent larvae and adult worm stages (*e.g.* L1/L2 or L2/L3), or to adjust the ratio of these directly in a microfluidic chamber. Also extraction of embryos from adult worm populations is reported.

Experimental

Materials

4-inch 550 µm thick Si and float glass wafers were obtained from the EPFL Center of Micro- and Nanotechnology (EPFL-CMI). GM 1075 SU-8 negative photoresist was purchased from Gersteltec (Pully, Switzerland). PDMS Sylgard 184 was acquired from Dow Corning (Wiesbaden, Germany). 1 mL borosilicate H-TLL-PE syringes were purchased from Innovative Labor Systeme (Stutzerbach, Germany). Microline ethylvinylacetate tubes with 0.51 mm inner and 1.52 mm outer diameters was bought from Fisher Scientific (Wohlen, Switzerland). L-Broth bacterial culture medium was obtained by adding 10 g Bacto-tryptone, 5 g Bacto-yeast, 5 g NaCl, in 1 L of H_2O. S-Basal was obtained by adding 5.85 g NaCl, 1 g K2 HPO4, 6 g KH2PO4, 1 ml cholesterol (5 mg/ml in ethanol), in 1 L of H_2O. S-medium was obtained by adding 10 ml 1 M potassium citrate pH 6, 10 ml trace metals solution, 3 ml 1 M $CaCl_2$, 3 ml 1 M $MgSO_4$ in 1L of S-Basal. S-Basal, L-Broth and S-medium were sterilized by autoclaving. Bleach solution was obtained by adding commercial bleach and NaOH (4 N) at a ratio of 3:2. All the chemicals used in S-Basal, L-Broth, S-medium and bleach solutions were purchased from Sigma-Aldrich (Buchs, Switzerland).

Worm preparation

A single colony of *E. coli* OP50 was used from the streak plate and inoculated into L-Broth. The inoculated cultures were shaken and grew overnight at 37°C. *E. coli* OP50 suspension was then ready for use as food source for *C. elegans. C. elegans* N2 wild type strains used for all experiments were obtained from Dr. Laurent Mouchiroud of EPFL's 'Nestlé Chair in Energy Metabolism' laboratory. The *C. elegans*

N2 strain was cultured at 20 °C on nematode growth medium (NGM) plates seeded with *E. coli* OP50. For synchronization, 10–15 reproductively active adults were transferred to a fresh plate seeded with bacteria every four days. The adults were allowed to lay embryos for 2–6 h. After a sufficient number of embryos was produced, all adult worms were removed from the plate. The embryos were then allowed to develop up to the desired larva stage or to adulthood before being used for a specific sorting experiment. Prior to the experiment, the synchronized populations were removed from the NGM plates by washing the plates with filtered S-medium; hereafter they were mixed according the desired sorting experiment requirements. Subsequently, the worms were gently washed twice by centrifugation and resuspension in fresh filtered S-medium. The density of worms was first counted by dropping 5 µL of suspension onto a glass slide. The density was the adjusted to 2 - 5 worm/µl by adding S-medium. A 1 ml syringe was filled with the worm suspension, and connected to the chip inlet. Worm loading was carried out by using a syringe pump (neMESYS, Low Pressure Syringe Pump, Cetoni GmbH, Germany). Before each loading experiment, the syringe was shaken or refilled to avoid worm sedimentation in the syringe. For sorting experiments, synchronized populations of L1, L2, L3 or L4 larvae, and adults were used.

Experimental design

The layout of our worm sorting device in shown in Fig. 1a. The device is built from a two-layer PDMS structure allowing fluidic control (red in Fig. 1a) and valve pressure control (green in Fig. 1a). Fluidic and control layer are separated by a deformable PDMS membrane. Two worm chambers C1 and C2 (length 5.8 mm, width 1.4 mm, height 150 µm) are connected by four parallel channels (length 2.4 mm, width 200 µm, height 50 µm). The chambers are large enough to accommodate hundreds of *C. elegans* worms. Worm populations are initially loaded into C1 through the fluidic inlet (In), whereas sorted populations may be extracted from the device through a fluidic outlet (Out). Size-selective sorting takes place during the transfer of the worm population from C1 to C2. For this purpose, the effective cross-section and shape of the four transfer channels is adjusted by means of a pneumatic control chamber (width 400 µm, height 60 µm) that is positioned perpendicular and on top of the fluidic transfer channels. It functions as an adjustable valve (V2 in Fig. 1a) that compresses all four fluidic channels simultaneously in a well-controlled way. A second valve (V4) has been introduced as backup of V2. Similar valves are positioned at the inlet and outlet of the chip (V1 and V3, respectively). In contrast to V2 (or V4), V1 and V3 are mainly operated in an open/(nearly)closed state in order to confine worm populations in the chambers for imaging and counting. Schematic cross-sectional views of the parallel arrangement of the four microfluidic channels with valve V2 (along the line A-A') and of chamber C2 (along the line B-B') are also shown in Fig. 1a.

A photograph of the whole PDMS chip (76 mm × 52 mm) with 8 independent sorting units is shown in the upper part of Fig. 1b. The middle part of this figure is a zoom showing a single sorting unit, in which the fluidic layer is emphasized by using a red dye solution and the valve pressure control chambers are filled with a green dye solution, respectively. Tuning of the cross-section of a single transfer channel by modifying the valve control pressure is demonstrated in three photographs in the lower part of Fig. 1b. The situations (i) to (iii) illustrate how the fluidic path (as revealed by the red colour) is progressively obstructed by increasing the valve control pressure values from 0 bar, 0.7 bar to 1.9 bar, respectively. At 0 bar, the transfer channel is fully open. For higher control pressures, the PDMS membrane blocks the central section of the transfer channel, leaving open narrow fluidic paths on each sidewall. Size-filtering of worm populations is achieved by accurately adjusting the cross-section of these residual paths.

Deflection of the PDMS membrane as a function of valve control chamber pressure was quantified by optical image analysis of pictures like the ones shown in the lower part of Fig. 1b (i,ii,iii). A series of top-view pictures of a red dye-filled transfer channel situated at valve V2, obtained using increasing pressure values from 0 bar to 1.9 bar to the valve control chamber, was first converted to 8-bit grey pictures. The membrane deformation profiles shown in Fig. 1c, i.e. the opening sections of the fluidic channel, were obtained by analyzing the grey level values along the cross-section line C-C' in the lower part of Fig. 1b. As observed in Fig. 1c, the width of the two fluidic paths on each side of the transfer channel can be reliably adjusted as a function of control pressure. This feature is used in our device to realize adjustable filters for size-dependent worm sorting.

Device fabrication

Two dedicated molds were used to fabricate each of the PDMS layers. The mold for the fluidic control layer was made by a two-step process. First, 50 µm deep structures were dry etch into a Si wafer (Alcatel AMS 200 SE) defining the fluidic transfer channels. Subsequently, a 100 µm thick SU8-100 layer was spin-coated on top to define chambers C1 and C2 resulting in a total chamber height of 150 µm. Likewise, the master for the valve pressure control layer was made by etching 50 µm deep structures into a Si wafer. Furthermore, a 25 µm thick PDMS membrane (base-to-curing agent ratio 10:1) was fabricated by spin-coating on a Si blank wafer and cured at 70 °C for 2 h. The two molds and the blank wafer were treated with trimethylchlorosilane (TMCS) in a vacuum desiccator to prevent adhesion of PDMS during the molding process. After air plasma surface activation, the PDMS valve layer was bonded to the PDMS membrane on the blank wafer. The assembly was peeled off and, following another air plasma activation step, fixed to the fluidic layer. Access holes for tube insertion were punched beforehand into the PMDS layers. Finally, the individual PDMS devices were bonded onto a cover glass slide for mechanical support. The four valve control channels were filled with deionized DI water to avoid pressure loss by air diffusion through the PDMS layers (a problem arising when air-filled chambers would be used). Valve pressure was controlled by syringe pumps and measured by pressure sensors (Pressure Measurement Module Qmix P, Cetoni GmbH) in the connecting tubes.

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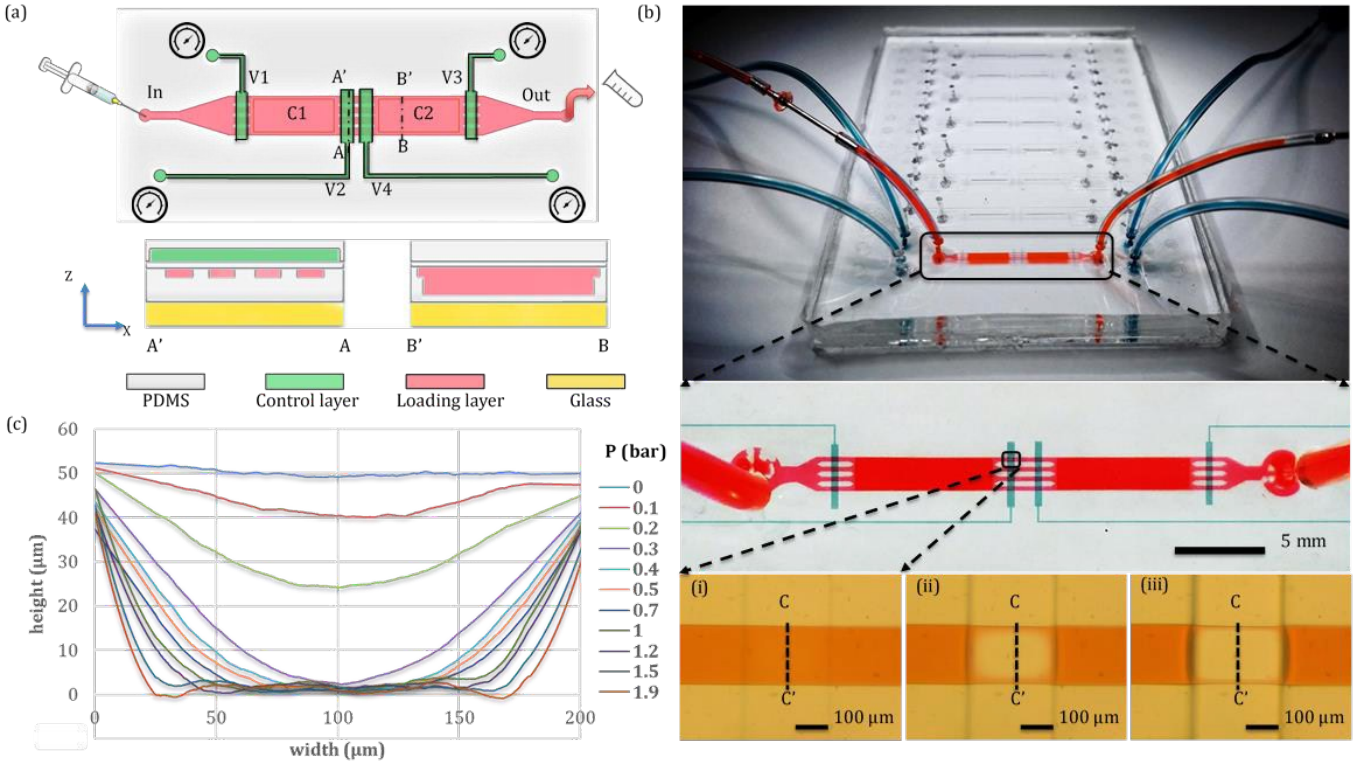

Fig. 1 (a) Schematic view of the worm sorting device (not to scale). The device is built from two PDMS layers, one for fluidic control (red) and one for valve pressure control (green), respectively. It comprises a single fluidic inlet (In), two worm chambers (C1, C2), one fluidic outlet (Out) and four valves used either as open/close valves (V1, V3) or as adjustable valves (V2, V4) for size-selective worm filtering. Mixed populations of worms are first loaded into C1. Subsequently, the desired portion of this population is transferred to C2 by adjusting the opening section of the transfer channels of V2 (V4 is not used here). Cross-sectional views of the four transfer channels of the valve V2 (along the line A-A') and of the chamber C2 (along the line B-B') are also shown. (b) Device photograph of the full chip with 8 individual worm selection units (one with tubes connected). The middle part shows the arrangement of fluidic and pressure control channels (filled with red and green dye solutions, respectively). The bottom pictures (i,ii,iii) illustrate the different states of a single transfer channel when using a valve control chamber pressure of 0, 0.7, and 1.9 bar, respectively. These images show how the fluidic path (red) is squeezed when pressure in the control channel is increased. (c) Deflection of the PDMS membrane (cross-section along the line C-C' in the picture of the lower part of Fig. 1b), defining the effective opening of the fluidic channel, as a function of control pressure measured by optical image analysis.

Results and discussion

Principle of size-selective sorting

The principle of size-dependent filtering in our device relies on the effective opening of the fluidic transfer channels which is adjusted by pressure control. Fig. 2a depicts schematically the cross-sectional view of one partially closed transfer channel due to the deflected PDMS membrane of valve V2. Assuming a circular cross-section of the nematode (diameter D_{worm}), we can set a threshold for passing or blocking worms of a certain size or age, respectively. The geometrical limit for passing the filter corresponds to the maximum diameter of a circle that can be inscribed in the residual fluidic opening path. In Fig. 2b, the red curve schematically represents the deflected membrane and the blue circle with radius O'C (center O') is the largest circle that fits in the opening. Here, C(x,z) is the point on the curve that has the minimum distance OC from the point of origin O. The diameter D_{pass} of the biggest worm which can still pass the filter can be determined as

$$D_{pass} = 2O'C = 2\frac{OC}{1+\sqrt{2}}. \qquad (1)$$

For size-dependent filtering, smaller worms ($D_{worm} \leq D_{pass}$) are transferred from chamber C1 to chamber C2, while bigger animals ($D_{worm} > D_{pass}$) are blocked by the membrane and retained in C1. This principle is explained in Fig. 2c. Different membrane deflections that would just block worms of a specific stage (or larger), the worm

diameter represented by corresponding circles, are schematically drawn. Based on this model, we estimated practical values for D_{pass} for the experimentally derived membrane deflection curves that were already shown in Fig. 1c. For this purpose, the experimental curves are expressed in (x,z) coordinates and the point C(x,z) can readily be found by minimizing the distance $(x^2+z^2)^{0.5}$ from the origin. Using eqn (1) we determine D_{pass} for different membrane deflections,

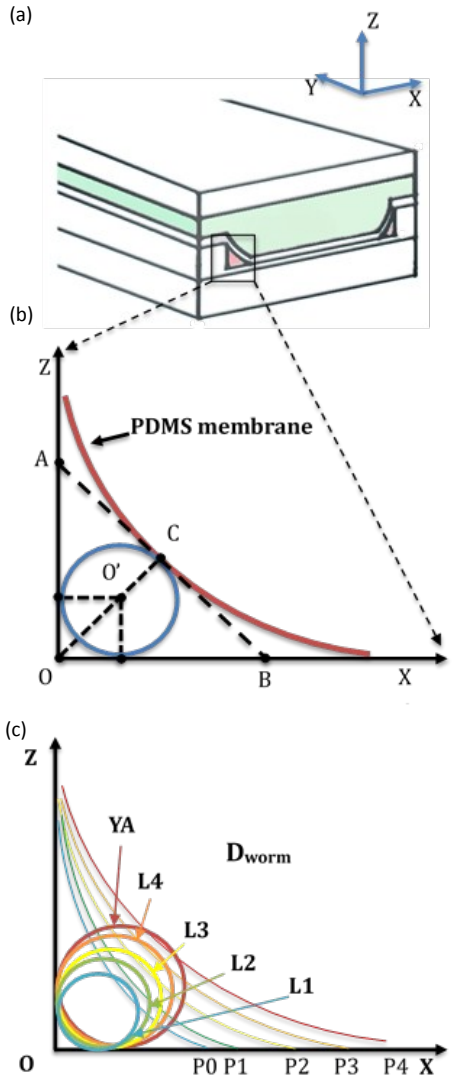

Fig. 2 Operation of the sorting device: (a) Schematic view of a single partially closed transfer channel due to the deflected PDMS membrane of valve V2. The pressure applied to the control channel (green) determines the opening section in the fluidic path (red). The compression is symmetric, leaving fluidic path openings on either edge of the channel. (b) Geometrical consideration of the fluidic filter defined by the deflected PDMS membrane (red line). The diameter of the biggest worm which can pass through the filter is given by D_{pass}=2O'C, corresponding to the maximum diameter of a circle (center O') that can be inscribed in the open fluidic path. (c) Graph explaining the mechanism of selective size filtering. Circles (diameter D_{worm}) indicate the worm stage (larvae L1 to L4, or young adults (YA)) that is just blocked by the filter position. The corresponding deflection of the PDMS valve membrane for different applied pressure values is schematically shown (P0 toP4, as reported in Table 1).

Table 1 Parameters for worm sorting: diameters for worms D_{worm} at different stages (larvae L1 - L4, and young adults) are indicated. D_{pass} is the maximum diameter of a circle that can be inscribed in the fluidic path opening for a given value P0-P4 of the applied external pressure on a valve control chamber (see Fig. 2b). Nomenclature: at PN, all larvae up to stage LN (with N=1-4) may pass through the filter. At P0 the valve is nearly closed, all larvae are blocked in chamber C1, only liquid can pass through. For P4 only adults are retained.

D_{worm} (µm)	L1	L2	L3	L4	YA
	11.7±0.2	**17.0±0.2**	**22.1±0.3**	**29.5±0.6**	**47.9±0.8**
Pressure (bar)	P0	P1	P2	P3	P4
	1.9	1.5	1.1	0.7	0.3
D_{pass} (µm)	10.4±0.3	13.0±0.4	18.9±0.3	24.1±0.3	33.3±0.2

i.e. as a function of the applied pressure and compare these values to the diameter D_{worm} of different larvae stages (L1 to L4) and young adult (YA) worms. Average values for *C. elegans* body diameters for each stage are reported in Table 1. These values have been determined by experimental measurements (on average about 30 worms for each population) using a digital microscope (KEYENCE VHX-700F) and correspond to values reported in literature [52]. The relationship between D_{pass} and pressure applied to the membrane was simulated by the Finite Element Method (FEM) for different PDMS membrane thicknesses and PDMS mixing ratios. Results are shown in the ESI† (Supplementary Movie S1 and Fig. S1). Values for D_{worm}, D_{pass} and the pressure values (P0-P4) to generate the corresponding membrane deflection are listed in Table 1. As an example, applying a pressure P3 of about 0.7 bar to the control channel, generates a membrane deflection that allows passing of all larvae stages up to L3 into chamber C2, but retains L4 larvae and adult worms in chamber C1.

Device operation and sorting procedure

Prior to worm loading, the device was prefilled with S-medium. Subsequently, a heterogeneous worm population was loaded into C1. For loading, a constant flow rate of 100 nl/s was applied. The corresponding valve configuration is shown in Fig. 3a. V1 and V3 are fully open during loading (no pressure applied to their control chambers). V2 is nearly closed by applying P0 to its control chamber, meaning that all larvae are blocked in chamber C1, but liquid can still pass. The population is then confined in C1 for imaging and counting by applying P0 to the control chambers of V1 and V2. For worm sorting, pressure on the control chamber V2 was changed to the required value for selecting worms of a certain size as listed in Table 1. The selected portion of the initial worm population was pushed through the transfer channels into C2 at a constant flow rate of 100 nl/s. V3 was set to retain the population inside C2 (Fig. 3b). After sorting and worm counting in C1 and C2, V3 was opened to collect the filtered worm population at the outlet (Fig. 3c). On the other hand, if desired, V3 can also be used as a second serial filter for a subsequent additional sorting step.

Larvae and adult worm sorting experiments

The initial heterogeneous population that is loaded into C1 may comprise embryos, larvae at all stages, and adult worms. Specific

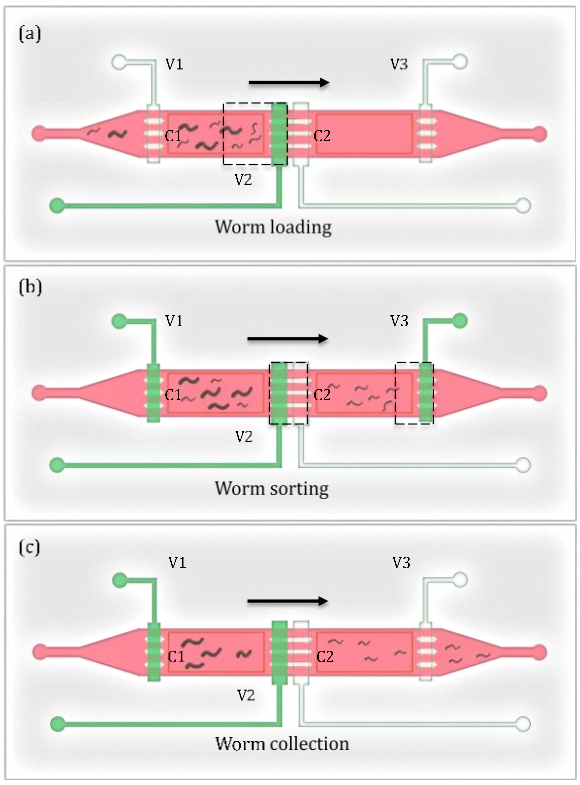

Fig. 3 Worm sorting protocol. (a) Worm loading: injection of a heterogeneous population of worms into chamber C1 (P0 is applied to the control chamber of V2, *i.e*. the transfer channels beneath are nearly closed and only liquid can pass to C2, V1 and V3 are fully open, V4 is not used/ its control chamber never pressurized in this experiment). Subsequently there is confinement in C1 (P0 applied to the control chambers of V1 and V2) and counting of larvae and adults is done. (b) Worm sorting: application of the required pressures P1-P4 to the control chamber of V2 (see Table 1) and transfer of the desired larvae population into C2. V3 is nearly closed by applying a pressure P0 to its control chamber, keeping all larvae inside C2 (eventually it can be left partially open by applying a pressure P1-P3, if used as a second serial filter). (c) Worm collection: the selected worm population is transferred from C2 to the outlet (V3 open).

sorting protocols are performed by adjusting the pressure settings of the different valves of the device, in particular V2 and V3. Our device allows versatile size- and age-dependent sorting resulting in populations comprising a unique larvae stage or only adults, or larvae of two or more different stages. With our approach, simple protocols may be applied to generate mixtures of larvae populations on-chip and different ratios of these. The versatility of the present device is demonstrated by a series of different sorting experiments. An outline of experiments that have been performed is shown in Table 2. The corresponding control pressure settings, applied to V2 and V3, respectively, and worm stages present in C1, C2 and at the outlet are summarized. The first and second part of the table explains extraction and isolation of larvae with unique size or of mixed populations in C2, respectively. In this case, an initial population containing larvae at all stages (L1 to L4) and adult worms has been loaded into C1. The third part of the table describes a protocol for adjusting ratios of larvae populations by 3 subsequent steps. For this experiment, a worm population that contains an equal amount of L2 and L1 larvae (ratio 1:1) was loaded into C1. With this protocol, first a population of L1 larvae is transferred from C1 to C2 (P1 on V2, P0 on V3). Then the outlet valve is partially opened (P1 on V3) to release a portion of this population. The amount of L1 larvae transferred to the outlet is controlled by applying flow through the device for a defined time span. In the particular case shown in Table 3, 50% of the L1 population in C2 have been removed. After closing the outlet, the whole L2 population may be transferred from C1 to C2 (P2 on V2, P0 on V3). The resulting population in C2 in a mixture of L2/L1 larvae at a ratio of 2:1.

Fig. 4 shows subsequent steps of a typical sorting experiment. As an example, we have chosen the extraction of L2 larvae from a mixed population of L2, L3 larvae and adult worms (no L1 and L4 are present in this case). Fig. 4a-c show photographs of the microfluidic device at important steps of the sorting experiment. Fig. 4a shows a picture of

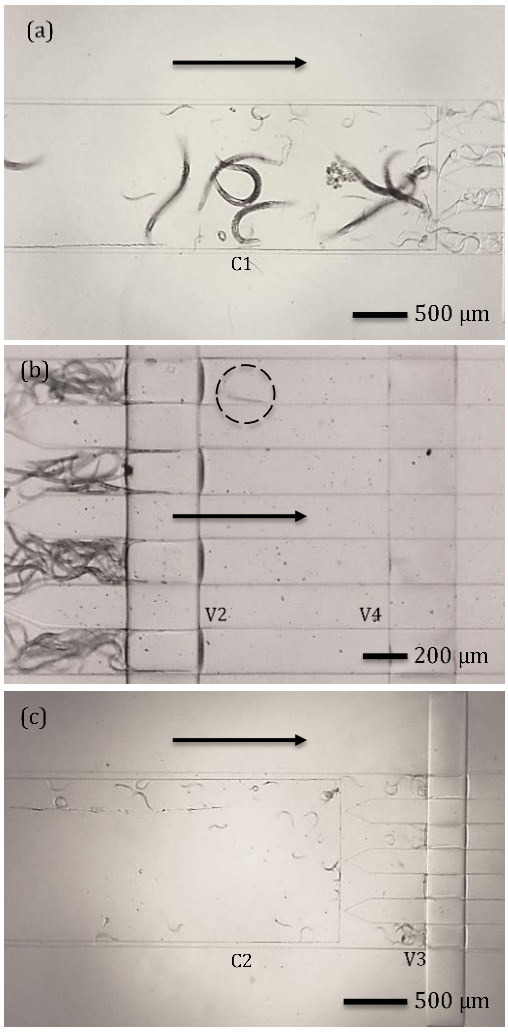

Fig. 4 Images of size-selective on-chip sorting of L2 larvae from a mixed population of worms: (a) a heterogeneous population comprising L2, L3 larvae and adult worms has been loaded into chamber C1 (V2 closed). For more convenient counting, the worms have been pushed in the vicinity of valve V2 (corresponding to the area defined by the dashed square in Fig. 3a). (b) Snapshot of a L2 larva passing through valve V2 where a pressure P2 has been applied (picture corresponding to the area defined by the left dashed square in Fig. 3b). Larger worms are blocked by V2. (c) Successful L2 sorting showing only L2 larvae in chamber C2 (picture corresponding to the area defined by the right dashed square in Fig. 3b).

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Table 2 List of possible sorting experiments: pressures applied to the control chambers of V2 and V3 to obtain various populations in C2 and to have specific worm populations transferred to the outlet are indicated; sorting of either single-stage larvae or mixed populations is possible. V1 is always in the nearly-closed state after loading (P0). The initial worm population loaded before sorting in chamber C1 is a heterogeneous mixture that may contain L1, L2, L3, L4 larvae and adults (A) worms. The third part of the table describes a 3-step protocol for adjusting ratios of larvae populations in C2 or at the outlet.

Sorting protocol	Valve state pressure		Population after sorting		
	V2	V3	C1	C2	Outlet
Single larva population in C2: Initial population in C1: L1, L2, L3, L4, A					
L1 sorting	P1	P0	L2, L3, L4, A	L1	-
L2 sorting	P2	P1	L3, L4, A	L2	L1
L3 sorting	P3	P2	L4, A	L3	L1, L2
L4 sorting	P4	P3	A	L4	L1, L2, L3
Mixed larvae population in C2: Initial population in C1: L1, L2, L3, L4, A					
L1&L2 sorting	P2	P0	L3, L4, A	L1, L2	-
L2&L3 sorting	P3	P1	L4, A	L2, L3	L1
L3&L4 sorting	P4	P2	A	L3, L4	L1, L2
L1, L2 & L3 sorting	P3	P0	L4, A	L1, L2, L3	-
L2, L3 & L4 sorting	P4	P1	A	L2, L3, L4	L1
Adjusting ratios: Initial ratio L2:L1 = (1:1) in C1					
1) Sorting of L1	P1	P0	L2	L1	-
2) Partial release of L1 from C2	P0	P1	L2	L1(50%)	L1(50%)
3) Mixing of L2 & L1 in C2	P2	P0	-	L2:L1 (2:1)	L1

the initial heterogeneous worm population in C1 (photograph corresponding to the area defined by the dashed square in Fig. 3a). For more accurate counting, worms may be concentrated in a smaller region of C1, corresponding to the field of view of the microscope, by pushing them gently towards valve V2 (Fig. 4a, P0 on V2). The number of L2, L3 and adult worms were counted one by one after closing the inlet valve V1. In order to selectively extract the L2 larvae population from C1, the valve filter V2 was then set to pressure P2. Using this pressure value, the effective width of the filter is 18.9 µm thus only L2 larvae having a diameter of typically 17 µm can pass through V2 (see Table 1). Fig. 4b is a snapshot showing a L2 larva passing through V2 (the photograph corresponds to the area defined by the dashed square in the middle of Fig. 3b). A video sequence of this event is shown in the ESI† (Supplementary Movie 2). Larger animals, *i.e.* L3 larvae and adults in this case, are retained in C1. During this operation, the outlet valve V3 was set to pressure P1 which corresponds to the threshold for retaining L2 larvae in C2. If present, L1 larvae, embryos but also small debris and bacteria clusters are removed from C1, pass through C2 and are transferred to the outlet. Immediately after the selective transfer, L2 larvae were confined in C2 by closing V3 for counting. It is noted that sometimes a larva gets partially blocked in a valve. However, such larvae do not pass the filter structure and retract back to chamber C1 after a short time, without significant impact on the sorting efficiency. Temporary L3 larvae blocking, for instance, can be seen in Supplementary Movie S2. To prevent clogging of the filter structure by young adults or adult worms, we take advantage of geometrical hindrance. As shown in Fig. 1a, worm chambers are three times higher than valve filters (height 150 µm vs 50 µm, respectively). Upon sorting of larvae groups from adults (diameter $\geq$ 50 µm) and by using flow rates in the lower range up to 100 nl/s, this height difference acts as a threshold that retains adult worms in chamber C1 at a certain distance from the valve channel (see Fig. 4a and Supplementary Movie S2). In this way, clogging can be safely avoided. After sorting of the larvae population, adults may be pushed towards and through the filter channels by increasing the flow rate (typically to 250 nl/s). Nevertheless, for reliable and reproducible worm sorting protocols, it is convenient to restrict the initial number of worms in chamber C1. This can be achieved by means of V1 and V2, as explained above. According to our observations, the number of adult worms loaded in chamber C1 should not exceed about 30 per experiment (i.e. 25 worms/µL for a chamber volume 1.2 µL) to avoid a 'steric hindrance'-like effect in sorting. Also the maximum total number for a mixed population loaded in chamber C1 should be around 80 worms (i.e. $\approx$ 67 worms/µL).

A photograph of the L2 population in C2 is shown in Fig. 4c (corresponding to the area defined by the right dashed square in Fig. 3b). Finally, the selected population may be collected at the outlet of the device. A significant advantage of our device is that by using adjustable filter valves, subsequent sorting steps of the same initial

worm population may be readily performed. For instance, in the present case, the L3 larvae population can be extracted from C1 by applying a pressure value P3 to valve V2, and P2 to valve V3, respectively. With this configuration, adult worms will be blocked in C1, L3 and L2 are transferred into C2 where only L3 are retained, whereas L2 pass to the outlet. In this way, serial separation of L2, L3 and adults, in this particular case, can be performed in a single step by applying a constant flow rate (typically 100 nL/s).

Sorting purity and efficiency

In order to evaluate the performance of our device, the number of larvae or adult worms was counted in chamber C1 and C2 before and after sorting. The results of separating L1 from L2, L2 from L3, L3 from L4, and L4 from adults are shown in Fig. 5. Overall sorting results were obtained based on the counting of individual worms of a certain population (total population sizes of about 250-350 worms; counting was done from 6-7 independent sorting experiments). According to a definition proposed by Ai *et al.* [50], we define the 'purity' of a worm population as the number of target worms in C1 or C2 divided by the total number of worms in that chamber. The 'efficiency' of the sorting process is defined as the number of target worms in C1 or C2 divided by the total number of target worms initially loaded in C1, before sorting. The sorting accuracy of the device for separating L1 from L2, L2 from L3, L3 from L4, and L4 from adults, respectively, is also reported in Table 3. Representative images of these sorting experiments are shown in the ESI† (Fig. S2). The device achieved an overall sorting purity and efficiency of close to 95-100 % for all larvae/worm groups. Throughput of the sorting progress mainly depends on the progress of the sorting experiment, i.e. on the actual larva/worm density in chamber C1, and to a minor extent on the larva/worm size. A typical sorting experiment lasts about 1 min. During this period throughput decreases from about 3.5 larvae/sec (i.e. ≈ 210 worms/min) or 3.0 adults/sec (i.e. ≈ 180 worms/min) at the beginning of a sorting experiment to about 1.0±0.5 worms/sec towards the end when the population of interest in chamber C1 declines. A detailed study on the evolution of the throughput during a sorting experiment is reported in the ESI† (Section S3 and Fig. S3). A video sequence of one sorting protocol is shown in the ESI† (Supplementary Movie 2).

Embryo extraction from adult worm populations

As explained in the introduction, synchronized populations of worms are required for many worm-based assays in order to eliminate variations of biological parameters due to age difference. Two methods are commonly used to obtain age-synchronized worm populations; they are both based on the sorting of embryos: (i) dissolving the adult worms by bleaching and simultaneous release of the embryos in the worm's body, (ii) collecting laid embryos directly from an agar plate. Here we show that we can perform with our device a very simple and efficient protocol to separate embryos from mixed adult worm populations directly in the liquid phase, thus rendering embryos immediately available for incubation. Moreover, if integrated in a microfluidic platform, embryos can be directly transferred to different modules for further processing or studies [53]. For preparing a mixed embryo and adult worm suspension, L1 worms were cultured in S-medium in a 96-well plate. *E. coli* OP50 was added as food source. After 4-5 days of culture at 20 °C in an incubator, the suspension mainly consisted of embryo-laying adult worms. Both, embryos and adult worms, were harvested and injected into C1 of the device. Selective transfer of embryos to C2 was achieved by adjusting the filter valve to a suitable opening cross-section (V4 was used here instead of V2). The typical embryo size is around 50 µm × 30 µm, whereas and adult worms have a diameter of larger than 50 µm. Size-separation can be easily achieved as embryos moves with longitudinal orientation through the filter. Appling a pressure of 0.6 bar to valve V4 results in an effective D_{pass} of 28.1 µm which is slightly smaller than the nominal value for a typical embryo diameter.

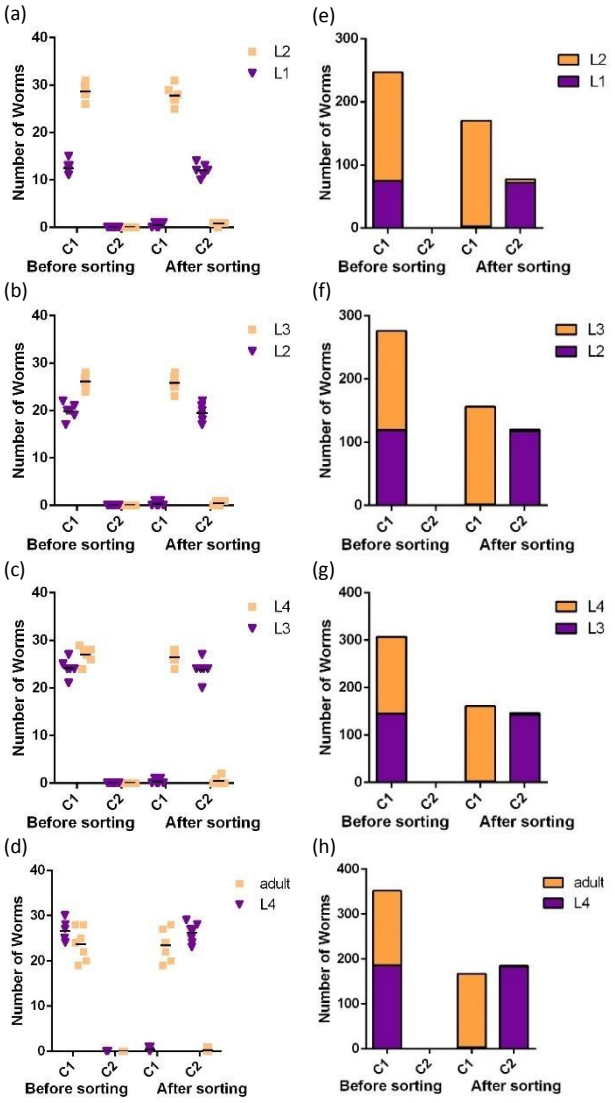

Fig. 5 Worm counting before and after sorting. In each experiment, a mixture of 2 populations of larvae and/or worms was introduced in chamber C1. Size-selection was obtained by applying the appropriate pressure to V2, after which the populations were counted in C1 and C2. In (a-d), the number of all individuals in a population counted in a single experiment is represented by a point and a triangle, while the average of similar experiments is represented by the dash. Summation of the counts done in all experiments is represented in (e-h). The two populations and the V2 pressure condition for the various sorting experiments are: (a, e) L1 and L2, pressure 1.5 bar; (b, f) L2 and L3, pressure 1.1 bar; (c, f) L3 and L4, pressure 0.7 bar; (d, h) L4 and adults, pressure 0.3 bar.

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Table 3 Larvae and adult worm separation purity and efficiency. Each value was determined from 6 or 7 independent experiments (shown in Fig. 5). Each experiment was performed with a total number of about 50 worms that were recovered from an agar plate and placed in C1. For every sorting experiment, two different populations (P1 and P2) were loaded into C1.

Populations loaded into C1	Average purity (%) of the populations in C1 before sorting		Average purity (%) of the populations in C1 and C2 after sorting		Efficiency (%) of the sorting	
P1 & P2	P1	P2	C2 (P1)	C1 (P2)	C2 (P1)	C1 (P2)
L1 & L2	31.5	68.5	93 ± 3	98 ± 2	96 ± 1	96 ± 1
L2 & L3	43.3	56.7	98 ± 2	97 ± 3	98 ± 3	98 ± 2
L3 & L4	45.1	54.9	98 ± 2	97 ± 4	98 ± 2	98 ± 3
L4 & Adult	48.9	51.1	98 ± 2	98 ± 2	98 ± 2	98 ± 2

However, as worm embryos may be slightly deformed, this is a suitable value for efficient sorting of embryos from adult worms, while still ensuring that no mechanical stress could damage the embryos during the separation process. Embryos may subsequently be recovered at the outlet of the chip and used for further culturing and bio-assays. Fig. 6a shows a snapshot of an embryo transfer from chamber C1 to C2. A video sequence of this event is shown in ESI† (Supplementary Movie 3). An adult worm is blocked by V4, whereas an embryo passes through (V2 fully open in this case). The result of embryo sorting from adults is shown in Fig. 6b,c. Before sorting, the ratio of embryos vs adults in C1 was about 3:2 for each measurement, corresponding to 10-30 adult worms and 20-50 embryos. After sorting, the purity of adults was 83±1% in C1 and 100% for embryos in C2, respectively, i.e. only embryos were found in C2. This observation demonstrates that a completely pure embryo suspension may be obtained with this method. The efficiency for embryo sorting was 85±1%. Eight independent experiments were used to evaluate the accuracy of embryo sorting. This result for embryo removal from C1 can be considered as very good, as strong adhesion of the sticky embryos to the chamber walls is commonly observed and represents an important issue in microfluidic manipulation. Sticky embryos also inevitably result in the formation of clusters inside the chamber. These groups are usually an issue for reliable chip operation and handling and separation of single embryos is very difficult. As can be observed in Fig. 6a, clusters of embryos are too big to pass through the filter of our device if adjusted by using the indicated pressure value for embryo sorting (p=0.6 bar). However, from the video shown in ESI† (Supplementary Movie 3), it is clear that fine-tuning of the opening size, *i.e.* fine-tuning of the flow speed and the related shear forces, plays an important role in disrupting clusters of embryos, and consequently improves the efficiency of embryo transfer. Sorting of eggs from L4 larva is still a challenge, since both have a diameter of approximately 30 µm. It is therefore difficult to extract a pure egg suspension from a natural worm population. Again, fine tuning of the flow rate might be a possible solution. On the one hand, one may take advantage of the high mobility of the L4 larvae to improve the filter selectivity with respect to eggs. On the other hand, eggs form clusters and easily stick to the surface of the PDMS chamber. Thus filtering at low flow rates, where only low shear forces are generated, may retain part of the egg suspension in chamber C1 whereas L4 pass through the filter into chamber C2.

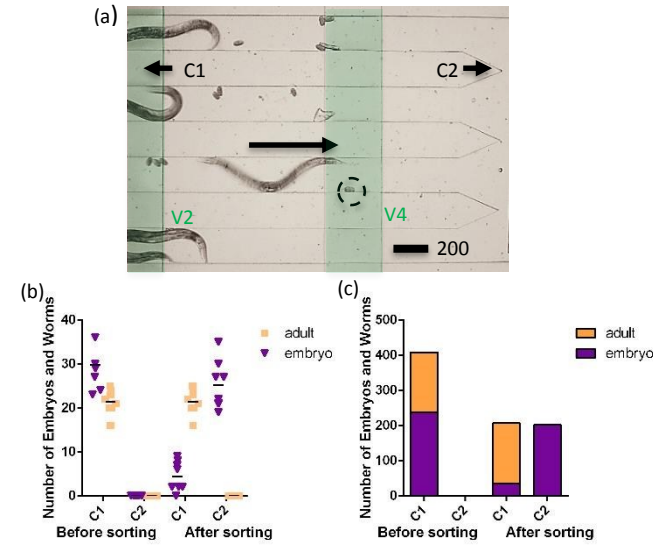

Fig. 6 Embryo and adult counting before and after sorting. (a) Photograph of an embryo that is present in V4 and transferring from chamber C1 to C2, whereas adult worms and embryo clusters are retained in C1. A pressure of 0.6 bar was applied to valve V4 during sorting (V2 open). The areas enhanced by the colour are for indicating the position of V2 and V4, respectively. (b) The number of embryos and adults in a population counted in a single experiment is represented by a point and a triangle, while the average of similar experiments is represented by the dash. In each experiment, a mixture of a population of embryos and adult worms was introduced in chamber C1. Summation of all counts done over the independent experiments is represented in (c).

Conclusion

C. elegans nematodes develop from hatching to reproductive adulthood through four larval stages (L1 to L4) that exhibit distinctive stage-specific features. For this reason, many biological studies on worms require populations of stage-synchronized animals for reliable interpretation of the results. In this work, we presented a new approach for size- and thus stage-specific sorting of heterogeneous worm populations. The sorting principle is based on the selective transfer of larvae through an adjustable microfluidic PDMS filter structure. Filter parameters, *i.e.* the effective cross-section of the fluidic path connecting two worm chambers, may be accurately adjusted through the externally applied control pressure. Compared with other manual, microfluidic or automated synchronization methods, this new approach appears to be very simple and highly efficient, yet is very flexible. Furthermore, since the device operation is merely based on geometrical parameters, *i.e.* filter opening section and animal diameter, instead of behavioral differences of worms, the sorting mechanism is by nature very reliable and reproducible. Also throughput, 3.5 worms per second, is higher than shown with electrotaxis-based sorting methods. Further upscaling of the throughput may be readily achieved by increasing the number of parallel filter channels on the chip. Another significant advantage is the versatility of the device. The same chip can be used for performing different sorting experiments with worms at all developmental stages, without changing the external dimensions of the microfluidic channels. Feasibility of worm sorting was demonstrated, among others, by separating groups of adjacent larvae stages (*e.g.* L1/L2) with close to 100% purity and efficiency in a single step. These results indicate that by performing several subsequent filtering step with appropriate filter settings, a natural mixture of worms could easily be separated into populations of the five developmental stages (L1 to L4 larvae and adults). It is possible to easily implemented on-chip such a serial filter structure by using the same fabrication process and design rules. On the other hand, we also demonstrated the possibility to generate on-chip controlled mixed larvae populations (*e.g.* L1&L2 or L1&L2&L3). Furthermore, for the time being, in some biological studies, e.g. social interaction pheromone-driven communication [54], different ratios of two worm population are used to study the influence between each other. There is no microfluidic device capable of adjusting the ratio of a given mixture of two worm populations. This option is available with our device by means of a simple microfluidic protocol.

For longitudinal developmental studies, an accurately age-matched cohort of animals is required, which can be obtained by isolating a batch of embryos that will hatch into a synchronized population. The adjustable filter structures of our device can be used as a powerful tool to isolate laid embryos from an adult worm population. In this way high purity embryo suspensions could be readily produced. Eventually, throughput for generating large synchronized populations might be significantly increased with respect to presently used methods, opening the way for more advanced and efficient drug screening assays, for instance. Beside larvae/adult and embryo/adult sorting, fine-tuning of the filter structures may provide a simple solution for any other possible size-dependent sorting protocol, such as mutant/mutant (e.g. lon-3 [55] and dpy-4 mutants [56, 57]), male/hermaphrodite and *C. elegans*/other nematodes (e.g. *P. pacificus*). A potential problem of size filtration is clogging due to particulates in the filtrate, in particular very sticky bacteria clusters, which hinders repeated or continuous operation of the device. This issue is greatly reduced by the use of filters with adjustable opening. We think that this new and simple sorting device has strong potential for future integrated microfluidic worm assays. For instance, the device can be easily integrated into a platform where stage- and/or age-synchronized populations, that are sequentially transferred to the outlet of the sorting chip, can be directly injected into bio-assay chip modules situated further downstream. This approach opens perspectives for advanced automated worm assays based on well-controlled and highly purified worm populations.

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