C. ELEGANS IMMOBILIZATION USING DEFORMABLE MICROFLUIDICS FOR *IN VIVO* STUDIES OF EARLY EMBRYOGENESIS AND INTESTINAL MICROBIOTA

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ABSTRACT

The genome of the nematode *Caenorhabditis elegans* shows far-reaching similarities to that of humans. *C. elegans* is therefore considered as a powerful model organism in biological research related to humans, for instance for the study of human diseases. Such studies are often based on high-resolution imaging of dynamic biological processes in the worm body tissues, requiring reliable immobilization of the nematodes. However, conventional immobilization methods may strongly affect physiological processes of the animal. Here, we report a new approach for on-chip immobilization of *C. elegans* nematodes, taking advantage of the elastic properties of polydimethylsiloxane (PDMS). We present two different microdevices, a micropillar array and a serpentine microchannel, respectively, both consisting of size-tunable PDMS structures that allow the same chips to be used for immobilization of worms at all development stages. Our microfluidic approach provides appropriate physiological conditions for long-term studies and enables worm recovery after the experiment. The performance of our devices is demonstrated by two different imaging experiments. The pillar array chip is used for *in vivo* live imaging of *C. elegans* embryo fertilization and early embryogenesis, whereas bacterial colonization of a worm's intestine was observed by means of the stretchable microchannel device.

INTRODUCTION

The millimeter-sized *C. elegans* nematode shares many fundamental cellular structures and biomolecular characteristics with more advanced organisms [1]. Many *C. elegans* mutants are available for biological research, which is especially important for genetic studies [1]. Its genome is very similar to that of humans (40% homologous), a major reason why *C. elegans* has become an attractive model organism in the study of human diseases [2]. For instance, in certain *C. elegans* models for neurodegenerative diseases, such as Alzheimer's, Parkinson's or Huntington's disease, disease progression can be assessed by monitoring cluster formation of specific proteins in the worm's body tissue [3]. Furthermore, *C. elegans* embryos are used as a powerful model to study the mechanics of metazoan cell division [4]. Optical interrogation of the worm's physiological state and of *in vivo* biomolecular processes often require high-resolution imaging for which the animal has to be securely immobilized. Any residual agitation may blur details of tissue fine structures or of dynamic molecular events, and thus reduce the information content of the assay. Conventional methods rely on anesthetic drugs [5], such as sodium azide, phenoxypropanol, and levamisole or mechanical immobilization with glue [6]. However, anesthetic compounds severely affect the animals' physiological state and usually preclude long-term imaging. Glue immobilization is a technically difficult procedure and does not allow recovery of the animals. Optical distortion from highly refractive glues may also be an issue. To circumvent such drawbacks, several microfluidic chips for worm immobilization and imaging have been developed in the past. Different techniques have been proposed, such as immobilization by a deflectable membrane [7], in tapered channels [8] or by means of a thermosensitive gel [9]. Nevertheless, either of these devices is associated with specific drawbacks, *e.g.* the complexity of the fabrication process, a certain lack of versatility, or the need of an external setup for thermal cycling of the device. In this paper, we propose two new microfluidic approaches for immobilizing *C. elegans* worms. Both take advantage of the elastic properties of a custom-designed PDMS structure and allow reliable and simple immobilization of worms at all development stages.

EXPERIMENTAL

Worm culture and bacterial strains

The *C. elegans* N2 wild-type strain used in this work was cultured at 20 °C on nematode growth medium (NGM) plates seeded with *E. coli* OP50. The same bacteria were also used as food source for *C. elegans* in the microfluidic chips. A single colony of *E. coli* OP50 was taken from a streak plate and inoculated into lysogeny broth (LB). The inoculated cultures were shaken and grown overnight at 37 °C. These suspensions were directly injected into the chip for worm feeding. For monitoring bacterial colonization of *C. elegans* by fluorescence microscopy, pGFPuv-expressing *E. coli* HT115 were used. The pGFPuv plasmid (Clontech Laboratories, Inc., USA), carrying a gene for a green fluorescent protein (GFP) variant optimized for maximal fluorescent response, was introduced into *E. coli* HT115 by electroporation. The pGFPuv-expressing *E. coli* HT115 strain was maintained in LB medium containing ampicillin (100 μg/ml) for selective growth.

DEVICE DESIGN AND OPERATION

We have designed two different devices for worm immobilization. One device is based on a 16 × 16 mm^2 PDMS micropillar array chip, mounted in a height-adjustable polymethylmethacrylate (PMMA) holder for applying compressive stress (Fig. 1 a,b). The pillars feature

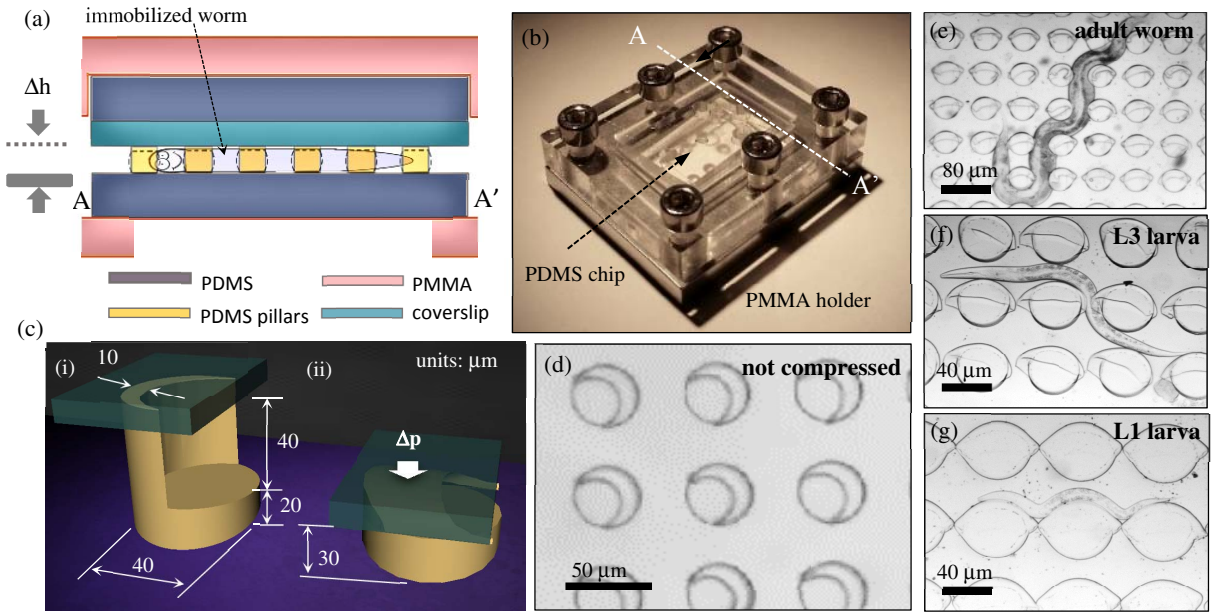

Figure 1: Micropillar-array device for C. elegans immobilization. (a) PDMS pillar array chip for worm clamping using a height-adjustable PMMA frame (cross-section). (b) Photograph of the device. (c) Schematic 3D view of a single pillar: (i) in the released state (height 60 µm) where the chip may accommodate adult worms or larvae; (ii) immobilization of adult worms is achieved by compressing the upper crescent-shaped part that collapses on the pillar base. (d) Top view of a portion of the full array showing nine crescent-shaped pillars in the uncompressed state. (e) Image of an immobilized adult worm (∅ ≈ 60 µm). Increasing the applied pressure also deforms the PDMS pillar base allowing immobilization of larvae at all stages, e.g. (f) L3 (∅ ≈ 25 µm) or (g) L1 (∅ ≈ 12 µm).

a specific dual-stage design with a 40 µm high foldable, crescent-shaped part on top of a 20 µm high, more stable, cylinder-shaped base part (Fig. 1 c,d). In the released non-compressed state, the total height and the lateral spacing of the pillars is 60 µm, thus arrays may accommodate freely moving adult worms (∅ ≈ 60 µm). Prior to worm loading, a 10 µl droplet of M9 buffer solution was introduced at the center of the micropillar array chip to provide a suitable liquid environment for the worms. Then, 10 to 20 individual worms were transferred from an agar plate and carefully placed into the droplet area. Subsequently, the pillar array was covered with a glass coverslip and placed in the PMMA holder for observation under the microscope. Finally, the distance of PMMA holder plates was adjusted via the incorporated screws, resulting in uniform compression of the PDMS pillar array. As not only the height but also the effective spacing between the compressed pillars decreases progressively with increasing pressure, the worms' movement may be restricted in a well-controlled manner and without excessively squeezing the worm bodies. This procedure may be applied to worms of all development stages. Examples for an adult worm, a L3 larva and a L1 larva are shown in Fig. 1e, f, and g, respectively.

The second device is a full PDMS chip that comprises a 15 µm wide and 40 µm high serpentine microfluidic channel. Worm suspensions are introduced through a single fluidic inlet, and eventually recovered at the outlet, respectively. The serpentine channel has 30 parallel channel sections with a length of about 10 mm each. This PDMS chip, which is stretchable by its nature, is mounted on a commercial tensile stage (Fig. 2a). Tensile stress is applied perpendicular to the parallel channel sections, by which the effective cross-section of these may be increased prior to worm loading (Fig. 2b). Subsequently, upon

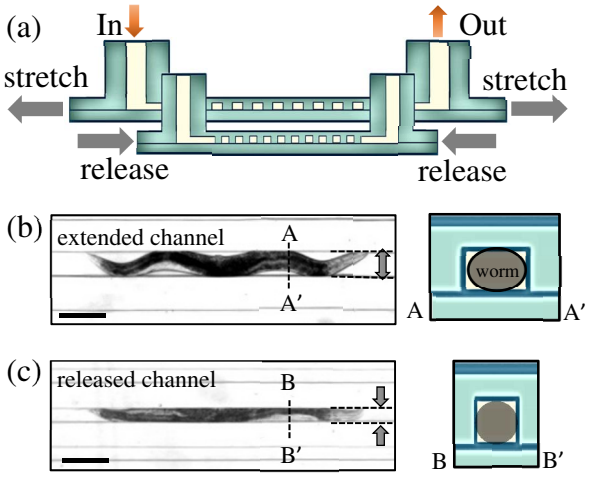

Figure 2: PDMS device with stretchable channel sections for immobilization and linear alignment of C. elegans worms. (a) The PDMS chip, which is attached to a tensile stage (not shown), comprises a single serpentine microchannel that receives the worm suspension. The cross-section in the figure reveals the parallel sections of the serpentine channel. (b) Image of an undulating adult worm during loading in the extended PDMS channel (width ≈ 80 µm, worm diameter ∅ ≈ 60 µm). (c) Immobilized, slightly compressed and aligned worm in the released channel structure (width ≈ 40 µm). Scale bar: 100 µm.

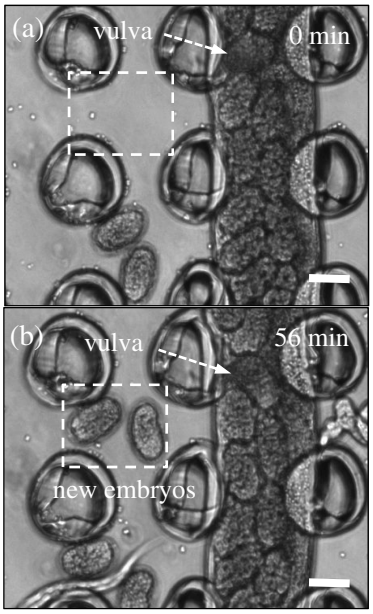

Figure 3: Live imaging (brightfield) of a C. elegans wild-type worm immobilized on the pillar array chip over long periods. (a) Detail of a gravid worm showing embryos in utero. (b) Two freshly laid embryos appeared on an image taken after 56 min. This observation demonstrates that egg laying occurs on-chip during immobilization, which is an indicator of good worm's health. The location of the worm's vulva is indicated. Scale bars: 30 μm.

release of tensile stress, worms are securely immobilized and aligned (Fig. 2c). Applied tensile stress values and/or geometrical dimensions of the channel can be adjusted for immobilizing worms at all development stages. For long-term immobilization (>30 min), *E. coli* is introduced during worm loading for food supply.

Devices used in this work were fabricated using standard soft lithography procedures. For the micropillar array device, the master mold was made by a two-step SU-8 on silicon process. First, the master structure for the foldable parts of the pillar array were created on the Si wafer by plasma etching (structure height 40 μm). Subsequently, a 20 μm thick SU-8 layer was spin-coated on the wafer and patterned to form the non-compressible pillar base structures. PDMS was poured onto the mold to obtain a 1 mm thick layer. For the stretchable device, a 40 μm high SU-8 serpentine structure was patterned on a silicon wafer and replicated in a 1 mm thick PDMS layer. The chip was plasma-bonded onto a second 1 mm flat PDMS layer to close the fluidic structures. All PDMS layers were cured at 80 °C for 1 hour (base to curing agent ratio 10:1). Beforehand, mold surfaces were treated with trimethylchlorosilane for 60 min to facilitate PDMS demolding.

RESULTS

Micropillar array chip

For each experiment, up to 20 worms were transferred onto the pillar array and securely immobilized. Worms were slightly squeezed by adjusting the height of the

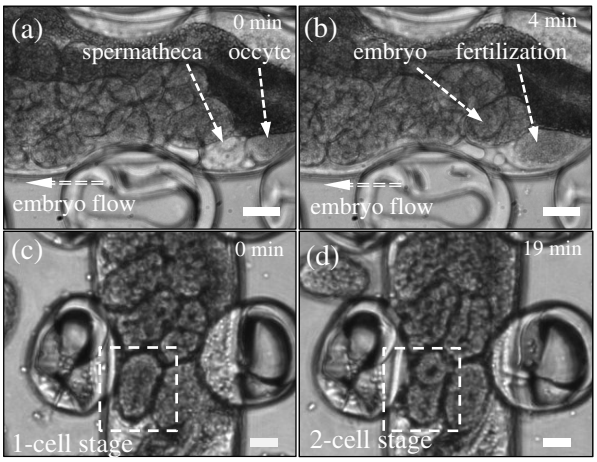

Figure 4: In vivo observation of early embryo development on the pillar-array chip. The images show a portion of the uterus of an immobilized gravid C. elegans hermaphrodite. All embryos slowly move to the left upon egg laying. (a) An unfertilized oocyte in the gonad is approaching the spermatheca. (b) After 4 minutes, the oocyte moved into the spermatheca. (c) A fertilized embryo in the one-cell stage appeared in the worm's uterus after passing the spermatheca (dashed frame). (d) The same embryo after 19 minute, i.e. after the first cellular division. Scale bars: 20 μm.

PMMA holder until residual movements of the body ceased. Nevertheless, this approach still allows agitation of the worm's head, an important requirement for maintaining adequate feeding conditions with *E. coli* that has been introduced on the chip prior to worm loading. As mentioned above, this immobilization protocol may be applied to worms at all development stages (Fig. 1e, f, and g). An on-chip egg-laying event was captured after immobilizing a gravid adult worm for about 1 hour on the pillar array (Fig. 3), demonstrating that this technique provides physiological conditions for normal worm behavior. Moreover, we have performed two specific *in vivo* high-resolution imaging experiments. First, an oocyte-to-embryo transition in a worm's uterus was recorded. Fig. 4 shows the process of fertilization occurring in the spermatheca located at the end of a gonad of an adult *C. elegans* hermaphrodite. Contractions of the oviduct force an oocyte to move towards (Fig. 4a), and finally into the spermatheca (Fig. 4b) where fertilization takes place. Subsequently, early embryogenesis may be directly studied *in vivo* in the uterus of the worm, starting from the first mitotic cellular division (~19 min after fertilization). Fig. 4c and Fig. 4d emphasize an embryo in the 1-cell and in the 2-cell stage, respectively.

Stretchable serpentine microchannel chip

Using the PDMS stretchable channel chip, bacterial colonization of a worm's intestine was visualized by fluorescent imaging. For that purpose, a group of adult worms was fed on agar plate with the pGFPuv-expressing *E. coli* HT115 bacterial strain. For on-chip imaging, worms were picked up manually from the agar plates, suspended in the bacteria solution and introduced in the serpentine channel for immobilization. Immobilization and on-chip

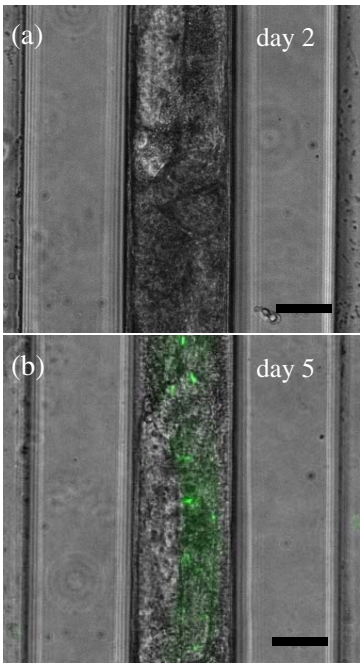

Figure 5: High-resolution in vivo imaging of the bacterial colonization of the intestine of an adult C. elegans worm. Worms were fed off-chip with a GFP-expressing E. coli bacterial strain and securely immobilized on a stretchable PDMS channel chip for imaging: (a) at day 2 of adulthood no fluorescent signal was observed; (b) at day 5 a GFP signal appeared everywhere in the worm's intestine, indicating that worms at an advanced age were not able to grind all of the consumed bacterial food. Scale bar: 40 μm.

imaging of the worm's intestine was performed at day 2 and at day 5 of adulthood. Worms were recovered after each experiment and placed again on an agar plate. At day 2 no significant fluorescent signal was observed (Fig. 5a). However, at day 5 a clear GFP signal appeared in the worm's intestine, generated by invasion of the worm's intestine with pGFPuv-expressing *E. coli* HT115 bacteria (Fig. 5b). Bacterial invasion of the worm's intestine could be due to an age-related abrasion of the worm's pharyngeal grinder, resulting in poor mechanical reduction of the bacterial food and incomplete digestion [10].

CONCLUSIONS

This article reports two new and simple microfluidic methods for on-chip immobilization of *C. elegans* nematodes, based on the reversible tuning of specific geometrical design parameters of a PDMS micropillar array and of a stretchable PDMS microfluidic channel structure, respectively. Immobilization of worms at all development stages under physiological conditions has been demonstrated, a feature that presents a significant advantage with respect to conventional immobilization methods. Each of our devices enables high-quality and long-term *in vivo* imaging of dynamic biological processes in *C. elegans* with unprecedented accuracy and simplicity. In particular, we demonstrated the feasibility of *in vivo* observation of oocyte fertilization and of early embryogenesis. Furthermore, bacterial colonization of adult worms was monitored by fluorescent imaging of the worm's intestine. We also anticipate that the linear on-chip alignment of a worm provided by the stretchable microchannel device will be particularly useful for advanced automated scanning imaging experiments.

ACKNOWLEDGEMENTS

The authors would like to thank the staff of the EPFL Center of MicroNanoTechnology (EPFL-CMI) for assistance in microfabrication, as well as Dr. V. Sorrentino (EPFL - Nestlé Chair in Energy Metabolism) who prepared the GFP-expressing *E. coli* bacterial strain. Funding of this work was granted by the EU Ideas program (ERC-2012-AdG-320404).

REFERENCES

[1] C. J. Kenyon, "The genetics of ageing", *Nature*, 2010, 464, 504-512.

[2] V. Sivagnanam and M. A. M. Gijs, "Exploring Living Multicellular Organisms, Organs, and Tissues Using Microfluidic Systems", *Chem. Rev.,2013,* 113(5), 3214-3247.

[3] M. Cornaglia, G. Krishnamani, L. Mouchiroud, V. Sorrentino, T. Lehnert, J. Auwerx and M. A. M. Gijs, "Automated longitudinal monitoring of *in vivo* protein aggregation in neurodegenerative disease *C. elegans* models", *Molecular Neurodegeneration*, 2016, 11(17).

[4] P. Gönczy, H. Schnabel, T. Kaletta, A. D. Amores, T. Hyman, R. Schnabel, "Dissection of cell division processes in the one cell stage Caenorhabditis elegans embryo by mutational analysis", *J Cell Biol.*, 1999, 144(5), 927-946.

[5] J. A. Lewis, C. H. Wu, H. Berg and J. H. Levine, "The genetics of levamisole resistance in the nematode *Caenorhabditis elegans*", *Genetics*, 1980, 95, 905–928.

[6] R. Kerr, V. L. Ram, G. B. P. Vincent, R. Y. Tsien and W. R. Schafer, "Active Currents Regulate Sensitivity and Dynamic Range in *C. elegans* Neurons", *Neuron*, 2000, 26, 583–594.

[7] T. V. Chokshi, A. Ben-Yakar, N. Chronis, "CO_2 and compressive immobilization of *C. elegans* on-chip*", Lab Chip,* 2009, 9, 151-157.

[8] S. E. Hulme, S. S. Shevkoplyas, J. Apfeld, W. Fontana, G. M. Whitesides, "A microfabricated array of clamps for immobilizing and imaging *C. elegans", Lab Chip,* 2017*,* 7, 1515-1523.

[9] J. Krajniak, H. Lu, "Long-term high-resolution imaging and culture of *C. elegans* in chip-gel hybrid microfluidic device for developmental studies*", Lab Chip,* 2010, 10, 1862-1868.

[10] C. D. Sifri, J. Begun, F. M. Ausubel, "The worm has turned--microbial virulence modeled in *Caenorhabditis elegans*", *Trends Microbiol*. 2005, 13(3), 119-127.

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