

# NIH Public Access

**Author Manuscript** 

Microsc Microanal. Author manuscript; available in PMC 2014 May 19.

Published in final edited form as:

Microsc Microanal. 2014 February; 20(1): 141-151. doi:10.1017/S1431927613014037.

## A Microfluidic-Enabled Mechanical Microcompressor for the Immobilization of Live Single- and Multi-Cellular Specimens

Yingjun Yan<sup>1,2,†</sup>, Liwei Jiang<sup>1,†</sup>, Karl J. Aufderheide<sup>3</sup>, Gus A. Wright<sup>1</sup>, Alexander Terekhov<sup>4</sup>, Lino Costa<sup>4</sup>, Kevin Qin<sup>1,2</sup>, W. Tyler McCleery<sup>5</sup>, John J. Fellenstein<sup>6</sup>, Alessandro Ustione<sup>7</sup>, J. Brian Robertson<sup>1</sup>, Carl Hirschie Johnson<sup>1</sup>, David W. Piston<sup>7</sup>, M. Shane Hutson<sup>5,8</sup>, John P. Wikswo<sup>5,7,8,9</sup>, William Hofmeister<sup>4,8</sup>, and Chris Janetopoulos<sup>1,2,8,\*</sup> <sup>1</sup>Department of Biological Sciences, Vanderbilt University, Nashville, TN 37232, USA

<sup>2</sup>Department of Cell and Developmental Biology, Vanderbilt University, Nashville, TN 37232, USA

<sup>3</sup>Department of Biology, Texas A&M University, College Station, TX 77843, USA

<sup>4</sup>Center for Laser Applications, University of Tennessee Space Institute, Tullahoma, TN 37388, USA

<sup>5</sup>Department of Physics and Astronomy, Vanderbilt University, Nashville, TN 37232, USA

<sup>6</sup>Vanderbilt Machine Shop, Vanderbilt University, Nashville, TN 37232, USA

<sup>7</sup>Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN 37232, USA

<sup>8</sup>Vanderbilt Institute for Integrative Biosystems Research and Education, Vanderbilt University, Nashville, TN 37232, USA

<sup>9</sup>Department of Biomedical Engineering, Vanderbilt University, Nashville, TN 37232, USA

## Abstract

A microcompressor is a precision mechanical device that flattens and immobilizes living cells and small organisms for optical microscopy, allowing enhanced visualization of sub-cellular structures and organelles. We have developed an easily fabricated device, which can be equipped with microfluidics, permitting the addition of media or chemicals during observation. This device can be used on both upright and inverted microscopes. The apparatus permits micrometer precision flattening for nondestructive immobilization of specimens as small as a bacterium, while also accommodating larger specimens, such as *Caenorhabditis elegans*, for long-term observations. The compressor mount is removable and allows easy specimen addition and recovery for later observation. Several customized specimen beds can be incorporated into the base. To demonstrate the capabilities of the device, we have imaged numerous cellular events in several protozoan species, in yeast cells, and in *Drosophila melanogaster* embryos. We have been able to document

Supplementary Material

*Corresponding author*. c.janetopoulos@vanderbilt.edu.

<sup>&</sup>lt;sup>†</sup>Both authors contributed equally to this study.

To view Supplementary Figures 1–7 and Supplementary Videos 1–8 for this article, please visit http://dx.doi.org/10.1017/S1431927613014037.

previously unreported events, and also perform photobleaching experiments, in conjugating *Tetrahymena thermophila*.

#### Keywords

microcompression; microfluidics; laser machining; pyramidal wells; *Paramecium tetraurelia*; *Saccharomyces cerevisiae*; polydimethylsiloxane

## INTRODUCTION

One major problem with biological light microscopy is the motility of living organisms. Keeping a living, wriggling specimen in focus and properly located in the field of view, especially for extended observation times, can be a major challenge. The need for immobilization of a specimen became more pressing after the development of contrastenhancement optical techniques (e.g., phase contrast, differential interference contrast) that allowed high-resolution observations of fine structures in living cells. The more recent advancements of genetically encoded fluorescent proteins and other *in vivo* labeling techniques have made the collection of adequate levels of fluorescent light even more critical. Many of these molecules are already dynamic in nature, so the added movement of the whole specimen can limit the ability to spatially and temporally resolve the localization of these components.

Many techniques to immobilize a microscopic specimen have been reported in the literature (Aufderheide, 2008). The specimen can be treated with an anesthetic or paralyzing chemical; or by various physical means, such as viscous media, or fibrous traps, or by methods of mechanical flattening and immobilization. All of these techniques distort the specimen's physiology and biochemistry to some degree, and they must be used carefully to avoid introducing artifacts into the events being observed. Some of these methods for trapping the specimen have the advantage of being quickly reversible: Once the specimen is released, the immobilizing agent is removed.

Mechanical immobilization techniques depend upon capturing the organism by carefully flattening it between two surfaces, both of which are typically transparent. While this can be done simply by drying a wet mount until the coverslip traps the specimen, the degree of control using this method is poor. The amount of time that can be spent examining the specimen is quite limited and recovery of the specimen can be difficult or impossible. Similarly, an agar overlay has been used to flatten cells to obtain better fluorescent images of membrane dynamics of cells (Fukui et al., 1987; Bretschneider et al., 2004), but recovery of the specimen becomes a major challenge. Sophisticated mechanical ways to achieve controlled trapping have been proposed through the years. Devices known variously as a rotocompressor, compressorium, or microcompressor have been developed and used. Several designs have been described (Aufderheide, 2008; Aufderheide & Janetopoulos, 2012), but all versions are essentially a coverslip mounted on a holder that can be precisely raised or lowered against the specimen to be trapped. Since the traditional design of a microcompressor involves solid glass components for the coverslip and slide, use of these materials limits the time a specimen may be trapped before gas exchange or fresh medium

might be needed. Similarly, the ability to change the fluid medium around the immobilized specimen is essentially nil. Because many of these devices were custom-machined in brass or steel, they were challenging and expensive to make, and often functioned only on specific types of microscopes. Although, one design (the Schaeffer rotocompressor) was commercially available in the 1950s, in general, these devices have never gained wide distribution or popularity among scientists.

With the advent of polydimethylsiloxane (PDMS)-based microfluidics, a new generation of immobilization devices has emerged (Lockery et al., 2008; Mannik et al., 2009; Westendorf et al., 2010; Wang et al., 2011; Yanik et al., 2011). While not as optically clear as glass, the PDMS polymer is transparent and is also gas permeable, so the specimen can remain visible and viable. Using standard PDMS-based soft-lithography, devices can also be made with any of a number of desired features and configurations (Whitesides et al., 2001). Several PDMS devices have been developed that contain chambers whose volumes can be adjusted by lowering or increasing the volume of air or liquid in a bladder above or below the specimen to be immobilized (Westendorf et al., 2010). Many of these devices have focused on trapping the nematode *Caenorhabditis elegans* (Mondal et al., 2012; Yang et al., 2013). These devices are typically not reusable, do not provide highresolution optics, may autofluoresce, and are often in a closed system that makes specimen loading difficult or manipulation of a specimen in a particular orientation nonexistent (McCormick et al., 2011; Shi et al., 2011).

We present here a mechanical microcompressor (MMC) that can work by itself or can be integrated with several microfluidic technologies. In essence, we have taken the basic structural design of the glass MMC and have incorporated the flexibility of PDMS-based microfluidics to fit the needs for trapping of a multitude of specimen types. This device was derived most recently from the Aufderheide rotocompressor design (Aufderheide, 1986; Aufderheide et al., 1992). Our precision platform is able to immobilize and hold living specimens ranging in size from microscopic bacteria to macroscopic fish embryos. There are several other design innovations in the device. One important aspect is that the metal components were designed to be fabricated on a computer-controlled milling machine (CCMM) and can be produced at a lower expense than traditional lathe machining. We also designed the device so that the coverslip is bowed significantly more for better trapping capability than previous versions. In addition, we designed the unit so that it could be used on either upright or inverted microscopes, a feature not available in earlier MMCs. As discussed above, we integrated PDMS-based microfluidics into the device. Most importantly, we have developed glass bases that have microchannels machined into them. Recent advances in femtosecond laser machining allow us to make three-dimensional (3D) channels in glass (Ke et al., 2005; White et al., 2008; Costa et al., 2011). These fluid channels can permit exchange of fresh medium while the specimen is being studied, and also permit introduction of drugs or other chemicals whose effects on the organism or cell can be observed in real time. We demonstrate the usefulness of these devices by immobilizing different types of organisms for extended times while sustaining their viability. Our devices also permit the possible recovery of the organism after observations and manipulations are completed.

## MATERIALS AND METHODS

#### Cell Culture

Two genetically marked strains of *Tetrahymena thermophila*, [CU 427 (Chx/Chx[cy-s]VI) and CU 438 (Pmr/Pmr[pm-s]IV)] were used for all experiments involving wild-type cells (Nanney & McCoy, 1976). Cells were grown to densities of 250,000–500,000 cells/mL in proteose peptone and yeast extract medium (Ng & Frankel, 1977), and starved in Dryl's solution for 18–24 h before mixing. In order to induce cells to be sexually reactive, equal numbers of cells of complementary mating type were mixed following starvation, according to established procedures (Martindale et al., 1982). For fluorescent imaging, Pdd1p-GFP was expressed in CU 427 and CU 438 mating cells and imaged using a 60× 1.49 NA lens ona Nikon (Tokyo, Japan) spinning disk confocal system and photobleached using a 405 nm laser from a Mosaic Unit.

*Paramecium sonneborni* (Aufderheide et al., 1983) and *Paramecium tetraurelia* were grown and imaged while in baked lettuce media and inoculated with *Klebsiella pneumoniae* (ATCC #27889) as a food organism (Aufderheide et al., 1999).

The *C. elegans* strain used (wdIs52, F49H12.4 ::gfp + unc-119) contained GFP integrated into its genome (Smith et al., 2010).

#### Imaging

A late third instar larva, labeled with Ubiquitous GFPEcadherin, was selected and washed in PBS. It was placed in the device in a drop of Halocarbon Oil 700 to allow for positioning. The upper coverslip was then lowered onto the larva until the mouth hooks were pinched into place with minimal movement allowed. The upper coverslip was rotated to orient the larva with eye discs visible and not obscured by larval glands. To further prevent movement for high-resolution imaging, a cold agar sample was placed directly on top of the slide. The images were taken on an inverted LSM 710 NLO laser scanning microscope (Zeiss Inc., Oberkochen, Germany), equipped with a Chameleon tunable laser (Coherent Inc., Santa Clara, CA, USA) for two-photon excitation microscopy. The lens used was an LD C-Apochromat 40×; 1.1 NA water immersion; W Korr M27 objective with long working distance (LD). A wavelength of 860 nm was used to excite the enhanced green fluorescent protein fluorescence and the emission was collected from 492 to 644 nm. Images in Figure 4a were enhanced to highlight morphology by cropping features by hand for false-coloring. While the animal imaged in Figure 4 was alive during imaging, it did not survive until the next day.

Appropriately staged *C. elegans* were picked from nematode growth media Petri plates and placed into a well of M9 buffer. The worm was then transferred by capillary aspiration from the well onto the viewing area of the compressor in a small droplet no more than 2  $\mu$ m in diameter.

Trapped *Saccharomyces cerevisiae* cells growing inside the compressor device and *C. elegans* were imaged with a Zeiss  $40 \times$  PlanNeofluar 1.3 NA oil objective at room temperature (22°C). For yeast observations, YPD medium was perfused at 0.5 mL/h. Images

in Figure 5 were captured on a Zeiss-Axiovert 200M microscope using SlideBook5 software (Intelligent Imaging and Innovations, Inc. (3I), Denver, CO, USA). FITC cubes for 3I Marianas workstation were used for GFP fluorescence.

#### Microfabrication

PDMS microfabrication was performed in the VIIBRE facilities as previously described (Jowhar et al., 2010). For the large horizontal channels in Supplementary Figure 6, toothpicks were used to mold the PDMS channels. All femtosecond machining of glass was performed at UTSI as previously described (Costa et al., 2011; Wright et al., 2012). Micromirrored wells were etched in silicon wafers (Seale et al., 2008), cut, and glued to the glass slide.

## RESULTS

#### Machining a Microcompressor for Immobilizing Single Cells

Several ongoing studies in our laboratory have required a device that could immobilize single cells, but we were also well aware that such a trapping device would be useful for investigators experimenting on other model systems. With this in mind, one of our design requirements was to construct a MMC where the brass components could easily be fabricated on a CCMM so that we could make several devices in one production run. Other microcompressor devices that have been used in the past for cell immobilization required a skilled machinist to make them, typically on a metalworking lathe.

The four brass components were all machined on the CCMM. The outer ring (OR) was glued to a standard  $2 \times 3$  inch glass slide (Fig. 1a). The inner ring (IR) threads into the OR. The compressor mount (CM) slip-fits securely within the IR; thus, turning the IR adjusts the height of the CM relative to the glass slide (G). The CM contains the brass coverslip compressor (CC), which is threaded into the CM and holds the 25  $\mu$ m wide  $\times$  0.18  $\mu$ m thick round coverslip. The CM and CC were machined so that when the CC is threaded tightly down, the coverslip's center bows downward slightly (Supplementary Fig. 1). This is a key feature of the device: The unique design of the CM ensures that the very center of the coverslip will be the first part to touch the centered specimen on the 12  $\mu$ m coverslip platform (CP). Bowing the top coverslip is critical because aligning two surfaces perfectly parallel to one another is nearly impossible. Without bowing, the edges of the 25  $\mu$ m coverslip and CP could make contact during use, and friction would prevent further rotation. Moreover, given that live cells are almost always in liquid, the bowed nature of the coverslip keeps the liquid in the center of the CP by capillary action. Parallel surfaces disperse fluid in more random directions by capillary action during compression and can drag the sample, shear it to pieces, or leave it stranded without liquid. The top coverslip is nestled into a machined recess in the CM (Supplementary Fig. 1). Because the coverslip is held from the bottom by the CM, a CP on the base is necessary for small specimens ( $<300 \,\mu m$  in height) to overcome the CM's thickness. The construction of the MMC is so precise that once the user determines where optimum immobilization of a specimen occurs, this spot can be marked; when a new, similarly sized specimen is put in the device, one can rapidly return to the optimal immobilization distance. It should be noted that the CM is released via a locking

mechanism from the IR so that the user does not have to completely unthread the IR to rapidly collect or change samples (Fig. 1b).

#### Machining a Microfluidic Base for Perfusion of Immobilized Specimens

Perfusion is not necessarily required for short observations or for single cells that require small volumes of media or buffer. However, studies involving long-duration time-lapse imaging depend upon the ability to add reagents or fresh media to immobilized specimens. To overcome the limitation of the standard MMC device as a closed system, we developed a base that incorporated microfluidic channels (Figs. 1b-1d). These channels were femtosecond laser machined into a 500  $\mu$ m thick fused silica chip bonded on the surface of the standard 2 × 3 inch glass slide. A second silica chip that makes up the CP had vertical ports machined through it that were connected to the underlying horizontal channels (Fig. 1d; Supplementary Fig. 2). Acrylic manifolds were attached by a silicon adhesive and supplied the solutions for perfusion. Thus, fresh media, or test solutions, can be added to the specimen while it is immobilized and under observation by use of the microfluidics channels.

#### Single Cell Immobilization

As examples of the utility of the system, mechanical compression was used to immobilize individual T. thermophila cells undergoing cytokinesis (Fig. 2a), and also conjugating pairs of cells throughout the stages of development during the ciliate sexual cycle (Fig. 2b). Cells could withstand compression for several hours, and cellular events could be monitored using high resolution differential interference contrast (DIC) or phase contrast microscopy. The micron precision of the device allows cells to be delicately trapped and flattened with little or no damage to the cell. The flattening of cells against the coverslip improved the optical performance of the microscopic system and provided higher resolution and detail. This configuration can provide images of events not previously documented. This can be seen, for example, with the elegant image of meiotic chromosomes at metaphase I in Figure 2b. The chromosomes and spindle microtubules are clearly visible. We imaged all of the major stages of conjugation in living T. thermophila, including the first live cell images of pronuclei moving toward each other to initiate and proceed to fertilization (Supplementary Fig. 3 and manuscript in preparation). We found that pronuclei could form spindle-like structures that could fuse end to end, side to side, or end to side (Supplementary Fig. 3 and not shown). In addition, we used the MMC to capture high resolution images of several other ciliates, including views of subcellular structures in *P. sonneborni* (Fig. 1c; Supplementary Video 1) and meiotic events during conjugation in P. tetraurelia (Supplementary Fig. 4). Ciliates appeared healthy as long as the contractile vacuoles were allowed to pump, and many cellular events could be monitored in real time (see Supplementary Video 1 for contractile vacuole pumping in P. sonneborni). We also used compression to flatten and obtain high resolution DIC images of the amoeba, Dictyostelium discoideum (Fig. 1d).

We have found that compression is useful for total internal reflection fluorescence microscopy (TIRFM). While flattened *D. discoideum* cells can move laterally, they are unable to move in the *z*-direction and can be stimulated with chemoattractants while under

gentle compression. Fluorescent imaging of plasma membrane dynamics can be observed using TIRFM (data not shown). Other researchers have used agar overlays to help keep a cell flattened against the coverslip for better TIRFM imaging (Fukui et al., 1987; Bretschneider et al., 2004; Matsuoka et al., 2012). Our system allows us to pick and choose which cells to compress and then flatten to the degree we deem appropriate.

We also acquired fluorescent images of conjugating *T. thermophila* cells expressing the protein Pdd1p fused to GFP, as shown in Figures 1e and 1f. The conjugant cell on the right underwent targeted photobleaching of its macronucleus. There was little or no recovery of the signal in this nucleus, suggesting that the turnover of this chromodomain protein is very slow. The MMC is a useful tool for observing high resolution cellular events in highly motile eukaryotic cells or for flattening cells to obtain organelles or structures in a particular focal plane for increased resolution and better visibility. In addition, as can be seen in Figure 1d, even bacteria can be trapped with the MMC. We have demonstrated that we can immobilize and then release individual bacteria (Supplementary Video 2).

#### Immobilization of Larger, Multicellular Organisms

The multicellular nematode C. elegans is a model system used to address fundamental questions in developmental biology and neurobiology. This worm is highly motile and difficult to image on a microscope. Several platforms have been fabricated using PDMS and have had some success at immobilizing these animals for microscopy and for high throughput analysis or mutant screening (Shi et al., 2011). The MMC that we developed can hold a C. elegans specimen motionless between the two coverslip surfaces (Fig. 3a) for extended times. We can immobilize embryos, worms of various developmental stages (L1-L4), and adults (Figs. 3a-3h). We were able to immobilize adult worms with developing embryos (Supplementary Video 3) and even adjust the compression in real time to trap the encased embryo after birth (Supplementary Video 4). Worms at several stages were trapped and imaged for at least 1 h, and then recovered, re-cultured, and scored for viability the next day. Of 21 worms immobilized, one was lost during the transfer stage and the rest were viable and highly motile the next day. Using perfused media, we also fully immobilized worms and held them for even longer times. The worm shown in Figures 3e-3h was immobilized continuously for 4 h; the images were acquired at the start and end of the immobilization time, and the worm remained in almost the exact same orientation. Shown in Figure 3 is the anterior and posterior neural network of the animal. This worm was still alive and motile upon release from this extended period of extreme compression. Going still further, we also immobilized worms overnight with perfusion of fresh medium and could keep such worms alive for up to 18 h, the longest duration tested.

To demonstrate the broad utility of the MMC, we also imaged the *D. melanogaster* eye imaginal disc in larval staged embryos. Keeping the larva still for imaging has proven extremely difficult for biologists. Previous work has attempted to view the eye imaginal disc in larger and more developed prepupae. This technique required anesthesia (1 mM levamisole) and the attachment of the embryo to a piece of double-sided adhesive tape. The animal was immersed in 100 mL voltalef oil to ensure an oxygen supply, and squished between bands of Parafilm by a coverslip (Escudero et al., 2007). Here, we took a third

instar larva labeled with ubiquitous GFP-E-cadherin, and placed the entire untreated specimen into the MMC. Using this technology, we rotated the larva (in the absence of anesthesia), and then obtained remarkable images of the imaginal eye disc during early morphogenesis (Figs. 4a-4c). The embryo in the figure was still alive at the end of imaging, but did not survive until the next day. We did not try to image *D. melanogaster* at any other stages, but this device should be useful for imaging larvae throughout development.

When properly adjusted, specimens can be gently flattened and immobilized in the MMC and observed for many minutes. The flattening can also improve the optical performance of the system by bringing the specimen into close proximity to the slide and the coverslip, making all surfaces perpendicular to the optical axis of the instrument. This device is not limited to cells or small metazoans; we have successfully cultured cross sections from developing murine intestinal tissue. The added microfluidic system capability provided perfusion for nourishment of the organ and the MMC kept the thin sections flat so that the entire section could be imaged in one "z" plane in overnight experiments (not shown). As shown with the *D. melanogaster* larvae (Fig. 4), the MMC can also be used to orient the specimen for better visualization of cell structures.

#### PDMS-Based Microfluidics Incorporated into the Mechanical Microcompressor

We created several disposable device bases with PDMS manifolds that gave us more flexibility for other studies in the laboratory and did not require femtosecond laser machining of glass (Fig. 5a; Supplementary Fig. 5). We envision the basic MMC as being useful to researchers at many institutions that now have access to microfabrication facilities (Primiceri et al., 2013). With this in mind we tested a variety of specimen platforms for use with C. elegans worms and other organisms. We used a high speed drill to incorporate 1  $\mu$ m holes through the glass base, and in some cases, through the coverslip specimen platform that was glued to the base. As shown in Figure 5a, a PDMS manifold could be plasmabonded to the base and connected to supply tubes driven by a peristaltic pump. This configuration was used on an inverted microscope. When used on an upright microscope, other manifolds were fabricated that had an L-shaped channel so that the tubes could be connected on the upper side of the unit, which did not interfere with the underlying condenser (not shown). Fluids readily traveled through the supply hole, across the compression zone, and into the exit port when we provided equal pressure on the supply side and suction on the return channel. We tried a number of PDMS channel configurations and posts to trap worms. We found that we could custom-design the PDMS bed, plasma-bonded to the base, to fit worms at various stages (Supplementary Fig. 6 and not shown). As illustrated in Figure 5b (Supplementary Video 5), we could also make an array of posts that would limit the lateral mobility of trapped worms. This allowed us to easily trap worms in a 3D volume with little or no physical flattening. We then could easily perfuse in media or bacteria to feed the worm (Supplementary Video 5) while it was immobilized. We also designed agar beds for worms, but found that worms were able to escape even high density agar beds. However, other specimens might not have the motile capacity to escape such a 3D confinement. With the MMC, one could design custom beds to trap an organism of choice.

We collected high resolution phase contrast images of *S. cerevisiae* cells growing in an MMC outfitted with a simple PDMS manifold. We gently trapped the cells and were able to image them for up to 5 h (the longest time attempted). The cells were observed to grow and bud (Figs. 5c, 5d; Supplementary Video 6) during this time. New buds were washed away by the current of the microfluidics system. This could provide an assay not only for visualizing the cell biology during yeast growth and budding, but also would allow for the collection of newly budded progeny in the return plumbing for genetic or proteomic analysis.

While we were able to trap large areas (>400 × 400  $\mu$ m) of ciliates and yeast (data not shown and Fig. 5b), we tested whether we could immobilize even larger fields by adding 5  $\mu$ m polystyrene beads. This would allow the user to set the height across a large area and to compress cells quickly without fear of over-compressing and damaging the specimen. We were able to confine the beads to small "z" volumes and trap large areas (>1.0 × 1.0  $\mu$ m) of beads, suggesting that precisely sized beads could be mixed with specimens for large-scale trapping and high throughput analysis (Supplementary Videos 7, 8).

Finally, we recently developed multiperspective imaging platforms formed by chemical etching of a silicon wafer, which can provide side views of a specimen (Seale et al., 2008, 2009). We used these silicon wafers containing micromirrored wells as the specimen platform and confined *T. thermophila* cells in a small volume that allowed us to image the cells from multiple perspectives (Supplementary Fig. 7). Since these mirrored wells can be fabricated with dimensions ranging from the nanoscale to hundreds of microns in depth and width, specimens of a wide range of sizes could be trapped and imaged from multiple perspectives by essentially lowering the ceiling down on the specimen. Given that the compressor coverslip bends and can compress a large area, we could potentially trap hundreds and even thousands of samples simultaneously with wells patterned in large arrays.

## DISCUSSION

The classical microcompressor devices that were developed during the 20th century are rarely found in use now. One major reason for their lack of wide utilization is that these devices are difficult to fabricate and are expensive. Only those researchers with ample resources, who had access to very good machinists and high precision machinery, could have them constructed (Spoon, 1978). Although the Schaeffer model was available commercially in the 1950s, other designs have never been produced by any of the major instrument makers. In this paper, we report the development of a MMC that was specifically designed so that it could be fabricated on a CCMM. This allowed us to make devices for much less machinist time than the earlier models. We have used several dozen units for prototype experimental and testing purposes, and we have now made them available for at-cost purchase and testing by the scientific community (http://

compressor.vueinnovations.com/). The brass machinery of the device is robust, and, with adequate care, should last for many years. The glass components are all commercially available and can be easily replaced if broken. The MMC has many research applications for use with different organisms and a variety of microscopes. It works on both inverted and upright microscopes, can reversibly immobilize specimens for short-or long-term studies,

and allows easy access to the specimen for recovery and re-culturing. It is also easy enough to use that it has been incorporated into an undergraduate cell biology laboratory course. In one laboratory period, for example, students can learn to use this device to successfully trap and examine living protozoa.

Several published reports have examined the capabilities of immobilization devices made of PDMS to trap specimens and cells (Chronis et al., 2007; Chung et al., 2008; Westendorf et al., 2010; Albrecht & Bargmann, 2011). These designs have PDMS ceilings or walls that can be gradually lowered or expanded by changing the volume in a channel or chamber that is adjacent to the specimen. This expansion can be controlled and provide trapping capacity. These designs have been shown with varying degrees of success to immobilize the nematode *C. elegans* (Shi et al., 2011). It should be noted, however, that many of these newer techniques use closed microfluidic devices that often require a sophisticated specimen loading process.

In an effort to make our MMC device even more flexible, we took advantage of our expertise in PDMS and glass-based microfluidic fabrication technologies to incorporate these newer microfabrication methods into our mechanical based platform of cell immobilization (Jowhar et al., 2010; Costa et al., 2011; Wright et al., 2012). We surmised that it would be valuable to modify the basic MMC design to produce a microfluidic device that allows easy cell and specimen loading and unloading, as well as reliable immobilization and microscope optical performance. Our MMC design, with added microfluidics, has been able to trap and hold bacteria, many species of protozoa, yeast, *D. melanogaster* larvae, and various stages of *C. elegans* worms.

In addition to qualitative observations, the MMC is useful for quantitative measurements of immobilized specimens as well as various other kinds of microsurgery and manipulations (Aufderheide et al., 1992, 1993). The open nature of the device's microfluidics system allow the experimenter to position and hold worms, for example, in a custom-designed trapping structure or "bed" microfabricated using PDMS (Fig. 5b; Supplementary Fig. 6). This setup physically confines the animal to a small volume and does not require much distortion for immobilization, which should limit any possible physiological artifacts generated by overcompression of the specimen. More delicate specimens can also be repeatedly trapped, quickly observed, and then released to reduce any possible stress that might be caused by mechanical compression.

We obtained high-resolution images of several protozoan species during vegetative growth and during their sexual cycles. Since it is otherwise nearly impossible to immobilize these highly motile cells, such studies have not been previously performed. We found that pronuclei assume a spindle-like structure before fertilization in *T. thermophila* and can fuse end-to-end, or in a variety of orientations, with the reciprocally exchanged pronuclei from the conjugation partner. In fixed specimens, these structures would appear to be mitotic or meiotic spindles, so visualization of this process in live cells was critical for furthering our understanding of the process of karyokinesis in these organisms. The movement of pronuclei before fertilization does not appear to be a random process, since pronuclei can clearly be seen moving toward one another before fusion. These pronuclei presumably track along

microtubules or other cytoskeletal elements to find their fusion partners. Once nuclear fusion occurs, the chromosomal materials from each pronucleus migrate toward one another and also mix, restoring the diploid number of chromosomes. The cells then immediately enter the first postzygotic mitosis.

Similar extended immobilization and media exchange experiments were performed on the yeast *S. cerevisiae*, which are typically about 10  $\mu$ m in diameter. This demonstrates that combining the MMC and microfluidics technologies permits use of the benefits of both capabilities. Yeast cells can be cultured continuously for hours, and potentially days, since budding off of new progeny could be observed. Because new progeny yeast cells are too small to be trapped, they are swept away by the microfluidic current and could be collected, cultured, and analyzed for proteomic or metabolic studies by mass spectrometry. In addition, the effluent from the microfluidic system could be collected during the life cycle of an organism or after various treatments. The low ceiling height of the device makes this idea quite attractive, because the fluid volumes used are tiny, and thus the signal to noise for identification of various released or secreted factors could be quite favorable.

We also took advantage of our ability to image specimens from multiple perspectives (Seale et al., 2008, 2009). We used a silicon wafer etched with an array of micromirrored wells as the specimen platform and confined *T. thermophila* cells in a small volume that allowed us to image them simultaneously from multiple perspectives. This has the potential to be used for a variety of applications, since custom-sized wells could be fabricated to trap a variety of sized specimens. The wells act more like cages than physical traps, so cells or small organisms could be confined to a small volume without actually compressing the specimen itself. Using the mirrored chambers, the specimen could then be imaged from several directions at once, which would have higher collection efficiency for fluorescence microscopy, since the arrangement of optics allows the capture of light that would otherwise typically be lost, and can be useful for single molecule studies or for molecules that do not fluoresce with high quantal efficiency. Since images are collected from the side, "*z*" information is also collected that is normally lost. This may prove useful for many of the new super-resolution microscope systems that are limited in their ability to gain as high a magnification in the "*z*" axis as they can in "*x*" and "*y*" axes.

Our ability to surface machine glass also allows us to make platforms that could have custom-designed "cages" for organisms to be trapped, as discussed above with the micromirrored wells. In addition, we could also perfuse each machined cage with microfluidic channels so that an individual specimen could be treated as desired with media, drugs, or chemicals and imaged over time. Such channels could be used for a variety of studies including the investigation of predator–prey relationships or host–pathogen interactions. The ability of organisms to be infected by pathogens or to interact with one another is often dependent on the physiological condition of both species. In this device, the environment could be manipulated and individual specimens could be followed over time to determine virulence of a pathogen, for instance. Many of the model systems immobilized here can be infected by human pathogens (Steinert & Heuner, 2005), or transfected with mutant human genes.

We also placed identically sized polystyrene beads in the microcompressor with the specimens and found that they could control the immobilization distance between the glass interfaces across very large areas of the imaging field. Addition of beads to the system could be useful for rapid immobilization, protection of the specimen from inadvertent crushing, and has potential applications for the high throughput analysis of compressed specimens.

Finally, an advantage that comes from mechanical compression is an increase in the size and stability of the "footprint" of cells for TIRFM imaging. During TIRFM, an evanescent wave excites fluorophores ;150 nm into the cell (Axelrod, 2001). This has become a very attractive technique for analyzing plasma membrane components and surfaceassociated signaling mechanisms and dynamics (Axelrod, 2003; Wazawa & Ueda, 2005). Because the excitation does not illuminate fluorophores deep within the cell, the signal to noise ratio is quite high within the area of illumination using this technique. Recent advances in the photon collection efficiency of electron-multiplying cameras have made the imaging of single molecules a reality and this is rapidly becoming an area of intense interest. One relatively simple extant technique, that has been used to flatten cells during TIRFM imaging, is an agar overlay (Fukui et al., 1987; Bretschneider et al., 2004). The agar overlay technique can be difficult to control or manipulate, but the MMC can be used for precision compression of cells for TIRFM in real time. This is particularly useful for imaging cells in response to an applied ligand. Many cells, such as the amoeba D. discoideum, will "cringe" and change their morphology when stimulated by chemoattractants. This can lead to a loss of fluorescence signal because TIRFM excitation is heavily dependent on the distance of the specimen from the coverslip. The gentle flattening of the cell in the MMC helps prevent this lifting and will provide a more accurate fluorescent signal, thus limiting artifacts.

## CONCLUSION

We have described a simple mechanical device for cell and specimen immobilization that has also been integrated with microfluidics and provides a wide range of uses for the investigation of cell biological functions for organisms and cells under microscopy observation. This MMC is useful for trapping motile specimens and also for the flattening of organisms for high resolution bright field and fluorescence microscopy. We have shown that a number of specimen platforms can be custom designed out of PDMS, lasermachined glass, or chemically etched silicon wafers for the trapping of a vast assortment of specimen shapes and sizes. Because of the precision flattening the device provides, it is particularly useful for delicate single cell immobilization, but will be valuable for a variety of microscopy studies and can also be used with numerous multi-cellular organisms.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

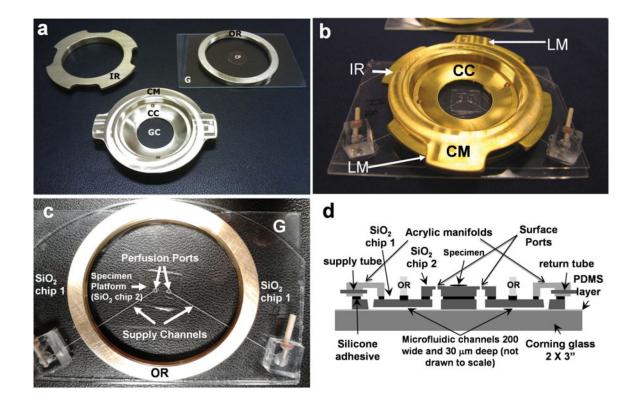
### Acknowledgments

The PDMS platforms would not have been possible without the use of VIIBRE's facilities. We would also like to thank the SYBBURE program sponsored by Gideon Searle and the Beckman Foundation, both of whom helped support L.J. We thank Cody Smith (Miller Lab) for use of his worms and for help in culturing worms. Thanks to Allison Price for critical reading of the manuscript and Dawit Jowhar for help with the videos.

## REFERENCES

- Albrecht DR, Bargmann CI. High-content behavioral analysis of *Caenorhabditis elegans* in precise spatiotemporal chemical environments. Nat Methods. 2011; 8(7):599–605. [PubMed: 21666667]
- Aufderheide K. Identification of the basal bodies and kinetodesmal fibers in living cells of *Paramecium tetraurelia* Sonneborn, 1975 and *Paramecium sonneborni* Aufderheide, Daggett & Nerad, 1983. J Protozool. 1986; 33(1):77–80. [PubMed: 3959010]
- Aufderheide K, Du Q, Fry E. Directed positioning of micronuclei in *Paramecium tetraurelia* with laser tweezers: Absence of detectible damage after manipulation. J Eukaryot Microbiol. 1993; 40(6):793– 796. [PubMed: 8292997]
- Aufderheide KJ. An overview of techniques for immobilizing and viewing living cells. Micron. 2008; 39(2):71–76. [PubMed: 17251031]
- Aufderheide KJ, Daggett PM, Nerad TA. *Paramecium sonneborni*, n. sp, a new member of the *Paramecium aurelia* species-complex. J Protozool. 1983; 30(1):128–131.
- Aufderheide KJ, Du Q, Fry ES. Directed positioning of nuclei in living *Paramecium tetraurelia*—Use of the laser optical force trap for developmental biology. Dev Genet. 1992; 13(3):235–240.
- Aufderheide, KJ.; Janetopoulos, C.; Méndez-Vilas, A. Immobilization of living specimens for microscopic observation. In: Méndez-Vilas, A., editor. Current Microscopy Contributions to Advances in Science and Technology. Formatex Research Center; Badajoz, Spain: 2012. p. 833-838.
- Aufderheide KJ, Rotolo TC, Grimes GW. Analyses of inverted ciliary rows in Paramecium. Combined light and election microscopic observations. Eur J Protistol. 1999; 35(1):81–91.
- Axelrod D. Total internal reflection fluorescence microscopy in cell biology. Traffic. 2001; 2(11):764–774. [PubMed: 11733042]
- Axelrod D. Total internal reflection fluorescence microscopy in cell biology. Methods Enzymol. 2003; 361:1–33. [PubMed: 12624904]
- Bretschneider T, Diez S, Anderson K, Heuser J, Clarke M, Muller-Taubenberger A, Kohler J, Gerisch G. Dynamic actin patterns and Arp2/3 assembly at the substrate-attached surface of motile cells. Curr Biol. 2004; 14(1):1–10. [PubMed: 14711408]
- Chronis N, Zimmer M, Bargmann CI. Microfluidics for *in vivo* imaging of neuronal and behavioral activity in *Caenorhabditis elegans*. Nat Methods . 2007; 4(9):727–731. [PubMed: 17704783]
- Chung K, Crane MM, Lu H. Automated on-chip rapid microscopy, phenotyping and sorting of *C. elegans*. Nat Methods. 2008; 5(7):637–643. [PubMed: 18568029]
- Costa LT, Terekhov A, Rajput D, Hofmeister W, Jowhar D, Wright G, Janetopoulos C. Femtosecond laser machined microfluidic devices for imaging of cells during chemotaxis. J Laser Appl. 2011; 23:042001–042006.
- Escudero LM, Bischoff M, Freeman M. Myosin II regulates complex cellular arrangement and epithelial architecture in drosophila. Dev Cell. 2007; 13(5):717–729. [PubMed: 17981139]
- Fukui Y, Yumura S, Yumura TK. Agar-overlay immunofluorescence: High-resolution studies of cytoskeletal components and their changes during chemotaxis. Methods Cell Biol. 1987; 28:347– 356. [PubMed: 3298995]
- Jowhar D, Wright G, Samson PC, Wikswo JP, Janetopoulos C. Open access microfluidic device for the study of cell migration during chemotaxis. Integr Biol (Camb). 2010; 2(11-12):648–658. [PubMed: 20949221]
- Ke K, Hasselbrink EF, Hunt AJ. Rapidly prototyped three-dimensional nanofluidic channel networks in glass substrates. Anal Chem. 2005; 77(16):5083–5088. [PubMed: 16097742]
- Lockery SR, Lawton KJ, Doll JC, Faumont S, Coulthard SM, Thiele TR, Chronis N, McCormick KE, Goodman MB, Pruitt BL. Artificial dirt: Microfluidic substrates for nematode neurobiology and behavior. J Neurophysiol. 2008; 99(6):3136–3143. [PubMed: 18337372]
- Mannik J, Driessen R, Galajda P, Keymer JE, Dekker C. Bacterial growth and motility in sub-micron constrictions. Proc Natl Acad Sci USA. 2009; 106(35):14861–14866. [PubMed: 19706420]
- Martindale DW, Allis CD, Bruns PJ. Conjugation in *Tetrahymena thermophila*. A temporal analysis of cytological stages. Exp Cell Res. 1982; 140(1):227–236. [PubMed: 7106201]

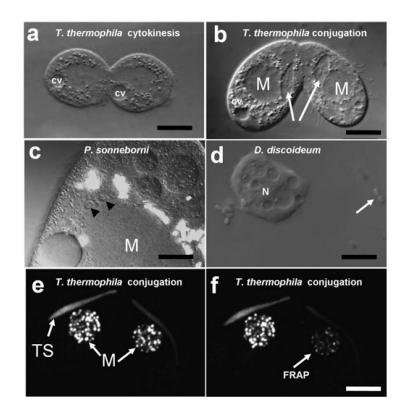
- Matsuoka S, Miyanaga Y, Yanagida T, Ueda M. Preparation of an imaging chamber for visualizing single molecules in living Dictyostelium cells. Cold Spring Harb Protoc. 2012; 2012(3):346–348. [PubMed: 22383649]
- McCormick KE, Gaertner BE, Sottile M, Phillips PC, Lockery SR. Microfluidic devices for analysis of spatial orientation behaviors in semi-restrained *Caenorhabditis elegans*. PLoS One. 2011; 6(10):e25710. [PubMed: 22022437]
- Mondal S, Ahlawat S, Koushika SP. Simple microfluidic devices for *in vivo* imaging of *C. elegans*, Drosophila and zebrafish. J Vis Exp. 2012; (67) doi:10.3791/3780.
- Nanney DL, McCoy JW. Characterization of the species of the *Tetrahymena pyriformis* complex. Trans Am Microsc Soc. 1976; 95(4):664–682. [PubMed: 828341]
- Ng SF, Frankel J. 180 degrees rotation of ciliary rows and its morphogenetic implications in *Tetrahymena pyriformis*. Proc Natl Acad Sci USA. 1977; 74(3):1115–1119. [PubMed: 403524]
- Primiceri E, Chiriaco MS, Rinaldi R, Maruccio G. Cell chips as new tools for cell biology—Results, perspectives and opportunities. Lab Chip. 2013; 13 (19):3789–3802. [PubMed: 23912640]
- Seale K, Janetopoulos C, Wikswo J. Micro-mirrors for nanoscale three-dimensional microscopy. ACS Nano. 2009; 3(3):493–497. [PubMed: 19309167]
- Seale K, Reiserer R, Markov D, Ges I, Wright C, Janetopoulos C, Wikswo J. Mirrored pyramidal wells for simultaneous multiple vantage point microscopy. J Microsc. 2008; 232(1):1–6. [PubMed: 19017196]
- Shi WW, Wen H, Lin BC, Qin JH. Microfluidic platform for the study of *Caenorhabditis elegans*. Top Curr Chem. 2011; 304:323–338. [PubMed: 21516386]
- Smith CJ, Watson JD, Spencer WC, O'Brien T, Cha B, Albeg A, Treinin M, Miller DM III. Timelapse imaging and cell-specific expression profiling reveal dynamic branching and molecular determinants of a multi-dendritic nociceptor in *C. elegans*. Dev Biol. 2010; 345(1):18–33. [PubMed: 20537990]
- Spoon DM. A new rotary microcompressor. Trans Am Microsc Soc. 1978; 97(3):412–416. [PubMed: 360576]
- Steinert M, Heuner K. Dictyostelium as host model for pathogenesis. Cell Microbiol. 2005; 7:307–314. [PubMed: 15679834]
- Wang J, Feng X, Du W, Liu BF. Microfluidic worm-chip for *in vivo* analysis of neuronal activity upon dynamic chemical stimulations. Anal Chim Acta. 2011; 701(1):23–28. [PubMed: 21763804]
- Wazawa T, Ueda M. Total internal reflection fluorescence microscopy in single molecule nanobioscience. Adv Biochem Eng Biotechnol. 2005; 95:77–106. [PubMed: 16080266]
- Westendorf C, Bae AJ, Erlenkamper C, Galland E, Franck C, Bodenschatz E, Beta C. Live cell flattening—traditional and novel approaches. PMC Biophys. 2010; 3(1):9. [PubMed: 20403171]
- White YV, Li XX, Sikorski Z, Davis LM, Hofmeister W. Single-pulse ultrafast-laser machining of high aspect nano-holes at the surface of SiO<sub>2</sub>. Opt Express. 2008; 16(19):14411–14420. [PubMed: 18794977]
- Whitesides GM, Ostuni E, Takayama S, Jiang X, Ingber DE. Soft lithography in biology and biochemistry. Annu Rev Biomed Eng. 2001; 3:335–373. [PubMed: 11447067]
- Wright GA, Costa L, Terekhov A, Jowhar D, Hofmeister W, Janetopoulos C. On-chip open microfluidic devices for chemotaxis studies. Microsc Microanal. 2012; 18(4):816–828. [PubMed: 22846851]
- Yang J, Chen Z, Yang F, Wang S, Hou F. A microfluidic device for rapid screening of chemotaxisdefective *Caenorhabditis elegans* mutants. Biomed Microdevices. 2013; 15(2):211–220. [PubMed: 23076545]
- Yanik MF, Rohde CB, Pardo-Martin C. Technologies for micromanipulating, imaging, and phenotyping small invertebrates and vertebrates. Annu Rev Biomed Eng. 2011; 13:185–217. [PubMed: 21756142]



#### Figure 1.

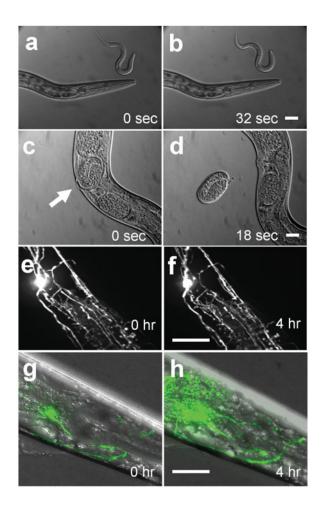
A Mechanical microcompressor device for immobilizing specimens. a: The main components of the microcompressor consist of a commercially available 2 inch by 3 inch glass slide (G), four precision machined brass pieces, and a machined aluminum tool (Supplementary Fig. 1). The brass pieces consist of a threaded outer brass ring (OR) cemented to the glass plate, a threaded inner ring (IR) that is screwed into the outer ring, and a brass coverslip mount (CM). The CM has a smooth outer face that slips into and engages the IR with two overhanging arms to form a locking mechanism (LM). The threaded inner face of the coverslip mount accepts the coverslip compressor (CC), and a machined step in the center of the mount creates a niche for a 25  $\mu$ m commercially available glass coverslip (GC). The unique design of the coverslip mount bends the coverslip so that the very center of the coverslip will be the first section to touch a centered specimen on the 12  $\mu$ m coverslip platform (CP). The platform can also be custom-designed for microfluidic control or for holding specimens in 3D volumes. b: Depicts an assembled unit, incorporated with microfluidics, ready to be placed on a microscope stage. Samples are loaded on the glass specimen platform at the center of the unit, after which the coverslip mount is inserted into the threaded assembly. Vertical adjustment of the top coverslip is made by rotating the ring assembly, engaging the fine threads of the two rings. c: The glass plate with outer ring from above incorporated with glass (SiO<sub>2</sub>) microfluidics. SiO<sub>2</sub> chip 1 has two 200  $\mu$ m wide and  $30 \,\mu m$  deep channels machined into it that each run from an acrylic manifold on the bottom left and bottom right of the image. These manifolds both have connectors for tubing that would carry fluid via a pump. The channels connect to SiO<sub>2</sub> chip 2, which serves as the CP and has a 30  $\mu$ m wide supply and return port for specimen perfusion. **d:** Schematic of the microfluidics from the side. SiO<sub>2</sub> chip 1 is adhered to the glass base by plasma-bonding to a

 $25-\mu$ m-thick layer of polydimethylsiloxane. The acrylic manifold and SiO<sub>2</sub> chip 2 are adhered to SiO<sub>2</sub> chip 1 by a silicone adhesive.



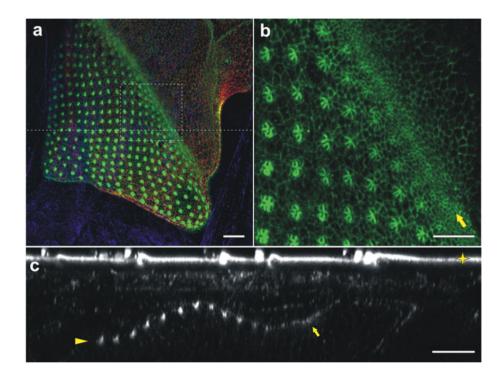
#### Figure 2.

Immobilization of living cells. Differential interference contrast (DIC) images of live cells using an Olympus  $100\times$ , 1.35 NA objective lens (Tokyo, Japan) (**a**–**d**). **a:** DIC image of *Tetrahymena thermophila* cell undergoing mitosis. Cell is just about to complete cytokinesis. Contractile vacuoles (cv) are labeled. **b:** DIC image of cell undergoing meiosis 1. Micronuclear chromosomes are at metaphase I (arrows), and are clearly visible, along with spindle microtubules. The macronucleus is labeled (M). **c:** Immobilized *Paramecium sonneborni* cell. Numerous organelles are visible, including the micronuclei (arrowheads) and the macronucleus (M). The contractile vacuole is in diastole on the lower left. See Supplementary Video 1 for movie of immobilized cell. **d:** DIC image of compressed *Dictyostelium discoideum* cell and *Klebsiella aerogenes* cell undergoing cell division (arrow). Also see Supplementary Video 2 for bacterial trapping. **e, f:** Confocal images of live immobilized *T. thermophila* cells using a Nikon  $60\times 1.45$  NA objective lens. The chromodomain protein Pdd1p-GFP localizes to foci with the macronucleus (M) during telophase I of meiosis. Telophase spindle (TS) is labeled. The macronucleus in the conjugant cell on the right is photobleached and undergoes FRAP. Scale bar is ~  $10 \, \mu$ m.



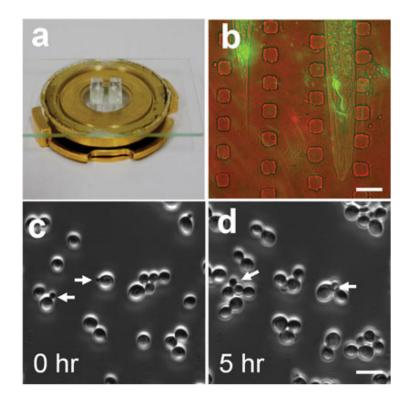
#### Figure 3.

Immobilization of the nematode *Caenorhabditis elegans* and visualization of the neural network. Compression of worms at various stages of development during M9 media perfusion in a device incorporated with SiO<sub>2</sub> microfluidics. **a:** Differential interference contrast (DIC) images of an adult and L1 worm imaged with a Zeiss 40×, 0.80 NA water immersion lens acquired on a Zeiss Axioskop 2 at time 0 s, and (**b**) 32 s later. **c:** DIC image of an adult worm with developing embryos at 0 s, and (**d**) after giving birth to the embryo (Supplementary Videos 3, 4). **e:** Projected image of PVD::GFP fluorescence from the neural network in posterior end of an adult worm at time 0 h, and (**f**) after 4 h of continuous immobilization using a 40× 1.35 NA DIC objective lens on an inverted Nikon spinning disk confocal system. **g:** Projected PVD::GFP and DIC image of the same worm near the anterior region of the worm at 0 h and (**h**) 4 h later. Scale bar is 20  $\mu$ m.



#### Figure 4.

In vivo imaging of *Drosophila melanogaster* eye imaginal disc by two-photon microscopy. **a:** Eye disc viewed through dorsal side of a third instar larva. The larva was gently flattened inside the mechanical microcompressor and imaged with two-photon microscopy using a  $40\times$ , 1.1 NA water immersion objective lens. All tissues labeled with ubiquitous E-cadherin GFP. **b:** High resolution image of box in (**a**). Undifferentiated cells (top right) contract their apical surface in a morphogenetic furrow (arrow) where they form a regular lattice of photoreceptor clusters. **c:** Cross-section (XZ-plane) of the eye disc and surrounding tissue [along dotted line in (**a**)]. Clusters of photoreceptors are apparent along the apical surface of the folded eye disc (arrowhead) behind the morphogenetic furrow (arrow). The disc lies under the larval epidermis (star). False coloring based on morphology is used to identify features: Green, Disc Proper; Red, Peripodial Epithelium; Blue—Surrounding Tissues and Muscles. Scale bar: (**a**) 20  $\mu$ m, (**b**) 10  $\mu$ m, and (**c**) 20  $\mu$ m.



## Figure 5.

Mechanical microcompressor (MMC) equipped with polydimethylsiloxane (PDMS)-based microperfusion. **a:** MMC with PDMS-based microperfusion. See Supplementary Figure 7 for a schematic of the device base. **b:** *Caenorhabditis elegans* trapped between an array of PDMS posts and being fed GFP-labeled *Escherichia coli* during perfusion (Supplementary Video 5). **c:** Phase contrast image using a  $40 \times 1.35$  NA lens of *Saccharomyces cerevisiae* at 0 h and (**d**) after 5 h of compression (Supplementary Video 6). Scale bar is 10  $\mu$ m.