

Scale-free Vertical Tracking Microscopy: Towards
Bridging Scales in Biological Oceanography
Supplementary Material

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1 Materials and Methods

2 1.1 Circular Fluidic Chamber Construction

3 The Circular fluidic chamber was custom fabricated from four parts: two spacer rings ma-
4 chined out of aluminum which were sandwiched between clear, scratch-resistant acrylic
5 sheets, resulting in an annular volume which was optically accessible from both sides (Fig.
6 S1B, C). The dimensions of the resulting annular volume was given by its inner radius R_i ,
7 outer radius R_o , and width W . Typical values of these parameters used in our experiments
8 were $R_i = 85 \text{ mm}$, $R_o = 100 \text{ to } 115 \text{ mm}$ and $W = 3 \text{ to } 6 \text{ mm}$ (Fig. S1D), resulting in
9 cross-sectional dimensions $L \times W$, where $L = R_o - R_i$. 100 % silicone adhesive (GE silicone)
10 was used to bond the various layers of the AFC so as to provide a biocompatible seal which
11 is also gas permeable to allow long-term experiments. Inlet and outlet ports made via luer
12 attachments (Cole-Parmer) allowed the chamber to be completed filled with fluid and also
13 allowed objects of interest to be introduced.

14 1.2 Motorized stages for tracking

15 The circular fluidic chamber was attached to a fine rotational stage using a high precision
16 shaft (Phidgets Inc.) and rotational bearings (Robotshop Inc., Canada) via a torsional-beam
17 coupler (Pololu robotics) (Fig. S1 A). The rotational stage comprised of a NEMA-11 stepper
18 motor mated to a 100:1 gearbox (Phidgets Inc., Canada), with a horizontal rotational axis
19 (Fig. S1 A). This arrangement allowed the chamber to be rotated with an angular resolution
20 of $19 \pm 1 \mu \text{ radians}$ per step, resulting in a tangential linear increment of $1.73 \pm 0.08 \mu \text{m}$ per
21 step at the center-line of the annulus. The rotation of the stage was measured using optical
22 encoders (Phidgets Inc.) with a resolution of $105.2 \mu \text{ radians}$ per pulse. The rotational
23 stage and bearings were mounted on height adjustable posts (ThorLabs, New Jersey, USA)
24 which were adjusted to ensure a horizontal axis.

25 The two other motion axes (x and y) were implemented using either standard off-the-

26 shelf translational stages driven by stepper motors (Haijie Technology Ltd., Beijing). In
27 our current implementation, the optical assembly was attached to the xy stages, such that
28 tracking in the xy directions was achieved by translating the optical assembly to follow the
29 object. Alternative designs are possible where motion compensation along all 3 axes (2
30 translation and 1 rotation) are applied to the circular fluidic chamber such that the optical
31 system can be fixed in the lab reference frame. Such an implementation may be better suited
32 when we one is interested in building advanced microscopy systems to work in conjunction
33 with our tracking method.

34 **1.3 Optical system**

35 We constructed a light microscope focused on either the 3 O' clock or 9 O' clock position
36 of the circular fluidic chamber such that rotational motion of the chamber resulted in ap-
37 proximately vertical motion (along the z direction) in the optical field-of-view (Fig. S1A).
38 The optical assembly was mounted on motorized translational xy stages for motion com-
39 pensation in the horizontal directions. The optical assembly consisted of a lens assembly
40 with an incorporated liquid-lens (Corning, Varioptic) which served as the imaging objective
41 (finite conjugate configuration), coupled to a CMOS camera (DFK 37BUX273, The Imaging
42 Source, Germany), capable of full resolution imaging (1440x1080 pixels) at 238 Hz, resulting
43 in an optical FOV of $2293\mu m \times 1720\mu m$. The imaging system was modular so that different
44 modalities could be interchanged. For tracking we used dark-field (DF) imaging using a ring
45 LED assembly situated on the opposite side of the fluidic chamber (Fig. S1). We primarily
46 used red (625 nm) (Fig. S12) LEDs to image since this does not induce phototactic behaviors
47 in most organisms. Images captured on the camera sensor were processed using a custom
48 image-processing pipeline implemented on a standard desktop CPU at rates of 100 Hz.

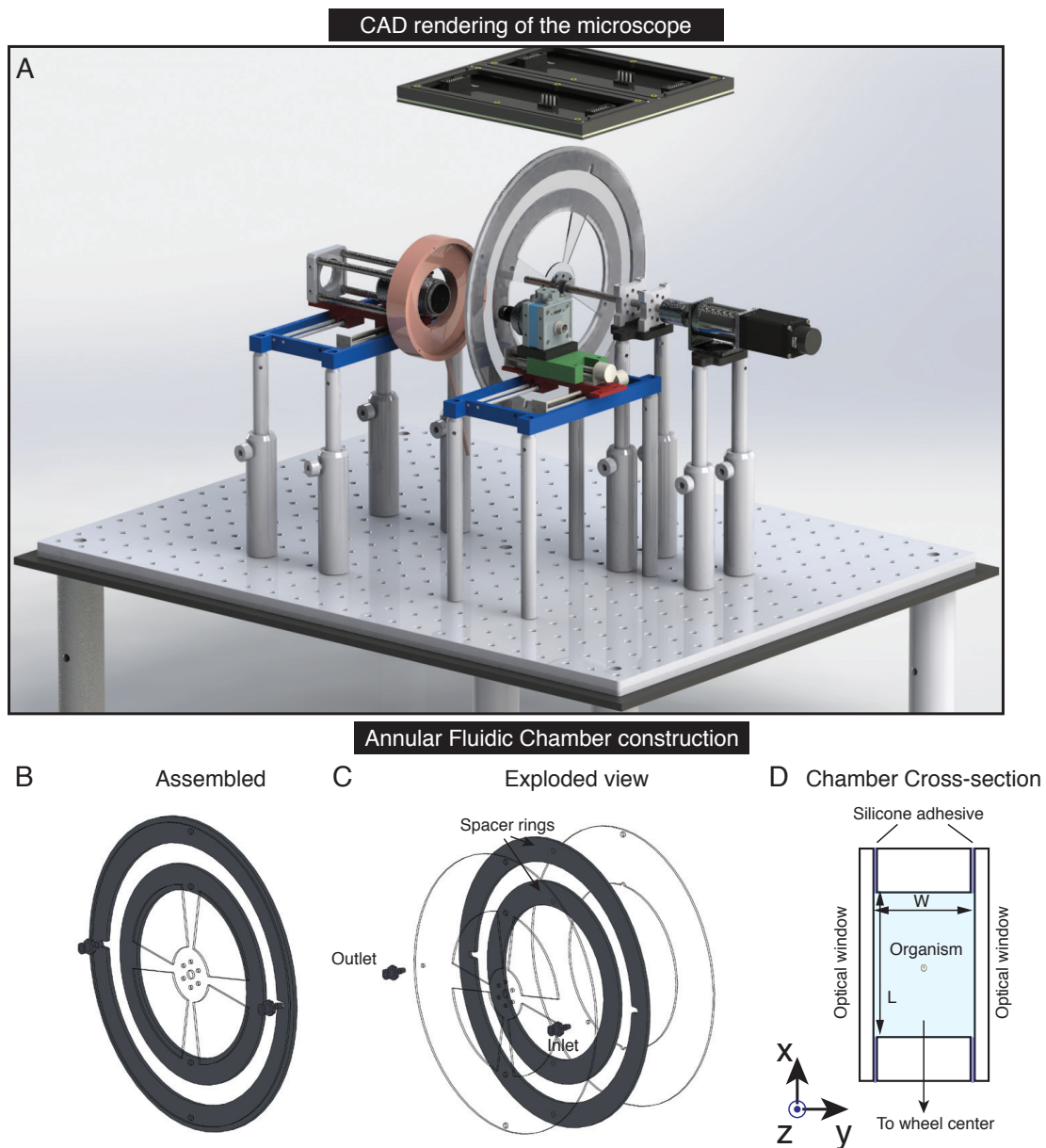


Figure S1 (A) CAD rendering of the tracking microscope, showing all major components as well as ambient light controller. (B) Assembled and (C) exploded view of the circular fluidic chamber. (D) Cross-section of the circular fluidic chamber.

49 1.4 Control system for tracking

50 Images for tracking were obtained from the CMOS sensor at rates of 100 Hz, and processed
51 using a custom image processing pipeline implemented using Python on a desktop
52 CPU (Fig. S2A). This pipeline consisted of separate organism trackers for lateral (xz) and
53 axial (y) positions. For lateral positions, an initial region of interest containing the object
54 was selected by the user, and this object was tracked in further frames using an open-source
55 object tracking algorithm available on OpenCV-Python [1] (Fig. S3). This algorithm was
56 robust enough to track the same object over a long times (1 day), even in the presence of
57 other similar looking objects, organisms and debris. As an alternative, to take advantage of
58 computers that have a GPU (Graphics Processing Unit), we utilized a hardware accelerated
59 object tracking algorithm [2] which allowed a combination of robust tracking and high frame
60 rates (up to 200 Hz). In all cases the output of the organism tracker was the lateral position
61 (x_{obj}, z_{obj}) of the object relative to the center of the microscope’s FOV. This output was fed
62 through a PID controller which in turn calculated the error signals that were sent to the
63 motorized stages (Fig. S2A).

64 1.4.1 Focus tracking

65 For estimating the axial position of objects, a separate focus tracking algorithm was de-
66 veloped. This used a liquid-lens (Caspian u-25H0-075, Varioptic, Corning) to rapidly scan
67 the focal plane and obtain image stacks at up to 30 volumes per second over a depth range
68 of 50-500 μm (Fig. S2B, C; Fig. S3). A focus measure of an image was estimated using
69 the image intensity variance [3, 4] (Fig. S3). A peak-finding algorithm was further used
70 to determine the focal plane position corresponding to the best focus and hence the ob-
71 ject’s position. This estimated position was fed to a proportional controller, with a tunable
72 gain and the resulting error signal was used to move the y -axis stage to follow the object
73 (Fig. S2C). We characterized the tracking performance of this method by tracking a 250 μm
74 bead mounted to a motorized stage that allowed prescribed motion of the bead along the

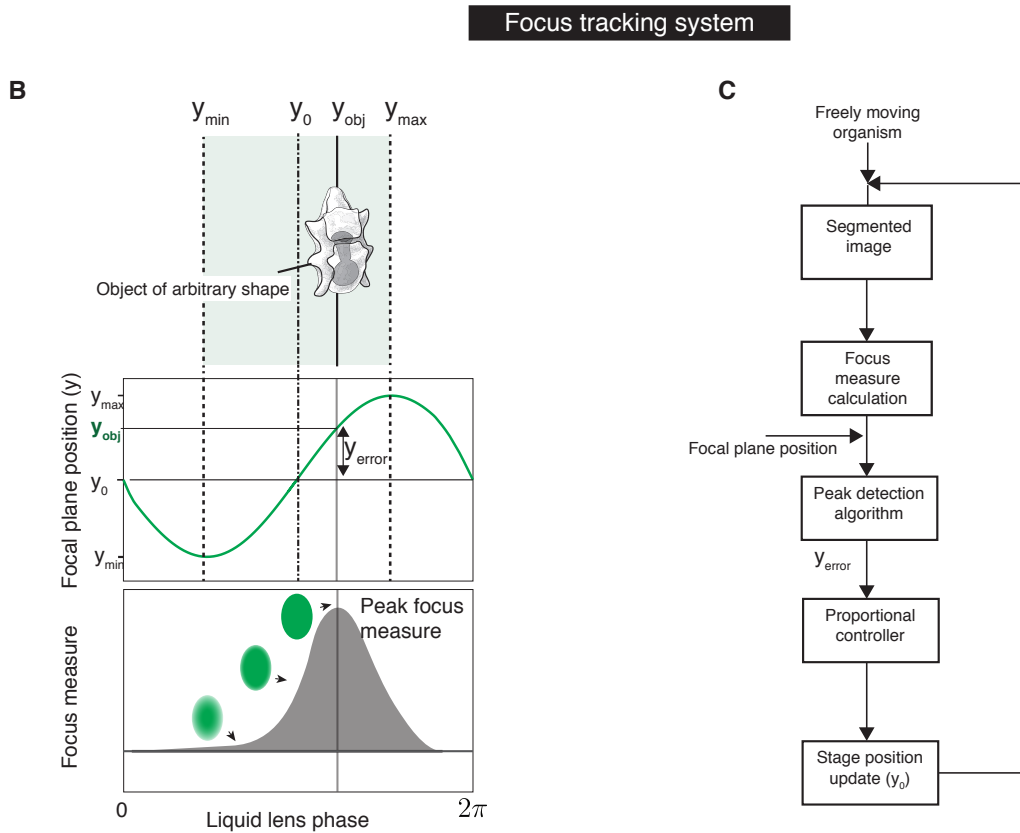
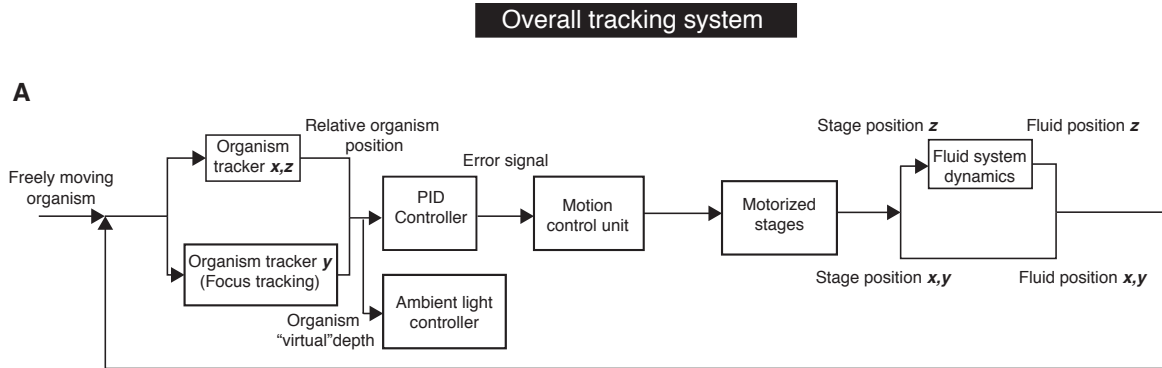


Figure S2 (A) Flow-chart of the tracking control system. **(B)** Focus-tracking methodology using a liquid-lens to sweep the focal plane of the microscope (Top, Middle), while calculating the focus measure (image variance) to locate the optimal focal plane that maximizes this focus measure (Bottom). **(C)** Flow-chart of the focus-tracking sub-system.

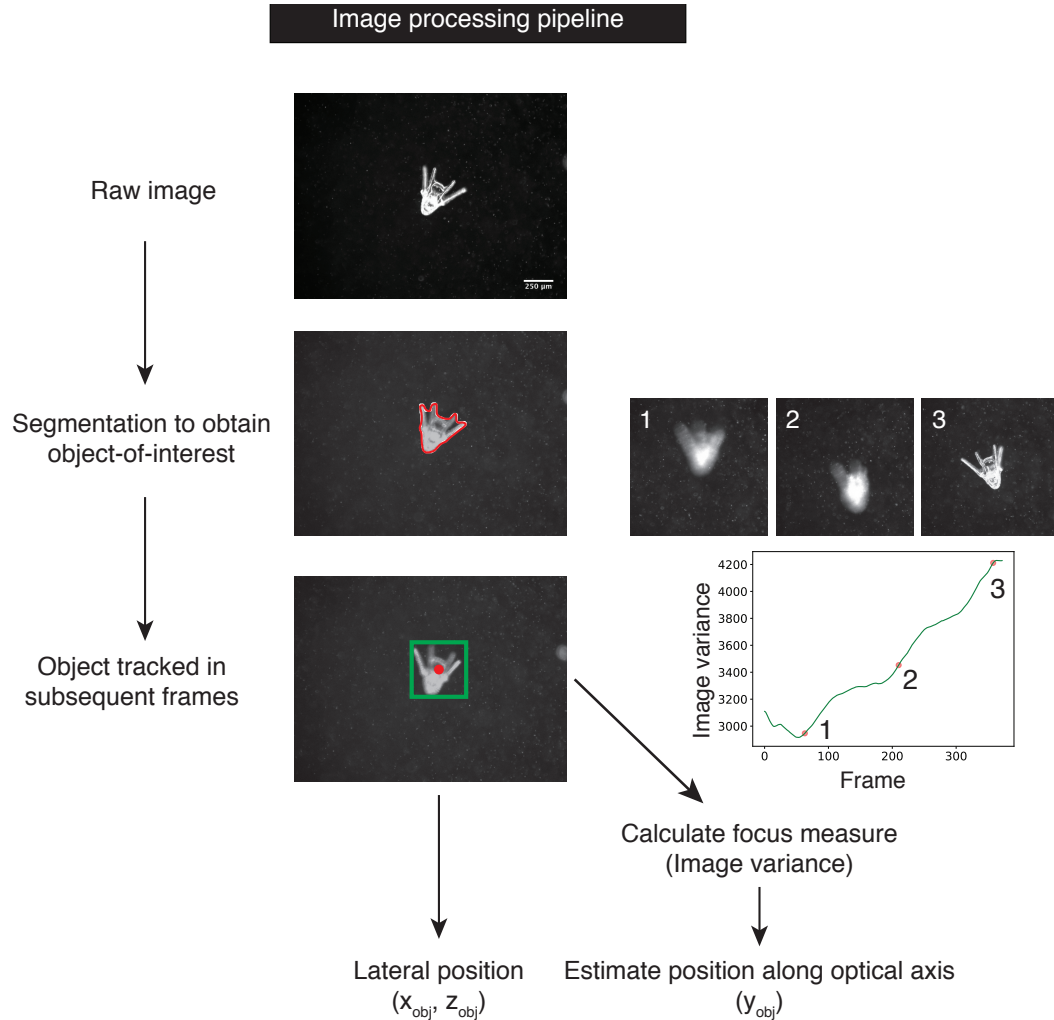


Figure S3 Image processing pipeline to initially obtain the object's centroid and track it in further frames. The initial location of the object is obtained by using a color-based threshold in HSV-color-space. In further frames the object is tracked using OpenCV's CSRT tracking algorithm [1], or the DaSiamRN algorithm [2]. The sample images are shown for Sand Dollar larvae (*D. excentricus*), which has a complex shape. Focus-measure (image variance) calculation for use in focus-tracking, showing both out-of-focus and in-focus images and their associated focus measures.

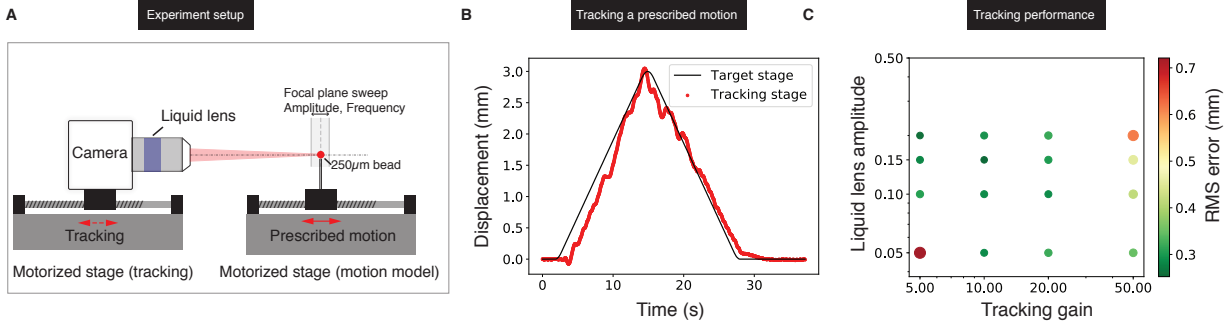


Figure S4 (A) Experimental setup used to characterize the performance of the focus-tracking system. This consists of two motorized stages, the first of which has a $250 \mu\text{m}$ bead attached, and executes a prescribed motion profile. The second stage is the tracking stage with the camera and liquid-lens optical system attached, which tracks the prescribed motion of the bead. (B) Displacement vs time plot for the stage with prescribed motion (black solid line), and the tracking stage (red dots) for liquid-lens amplitude of $100 \mu\text{m}$ and proportional gain of 10. (C) Characterization of focus-tracking performance showing a plot of the root-mean-square (RMS) error between the tracked and tracking stage as a function of the liquid-lens amplitude and the gain of the proportional controller. By an optimal choice of the lens amplitude and controller gain, RMS errors less than the tracked object's size could be achieved.

75 optical axis (Fig. S4A). This bead was then tracked using our focus tracking strategy by
 76 obtaining volume scans with the liquid-lens and translating the optical assembly to track the
 77 object (Fig. S4B). We characterized the tracking performance as a function of the scanning
 78 amplitude of the liquid-lens and the tune-able gain, and found an optimal range for these
 79 parameters (see Fig. S4C).

80 The error signals for all three-axes were sent to a Motion Control Unit which was a
 81 Arduino-Due microcontroller (Arduino). The microcontroller, in turn, was used to calculate
 82 the motion profiles for the motorized stages. These signals were sent to a dedicated stepper
 83 motor driver for each motorized stage axis (Big Easy Driver, Sparkfun) which used the
 84 Allegro A4988 stepper driver chip. The positions of the stages were measured using rotary
 85 optical encoders (HKT22, Phidgets Inc.) with a quadrature resolution of 600 counts per
 86 revolution.

1.5 Loading of the circular fluidic chamber and ensuring thermal equilibration

Before experiments the fluidic chamber was passivated by filling it with 5 % BSA solution (Fisher Scientific) and allowing it to sit for 1 hour. After this treatment the fluidic chamber was rinsed twice with the standard solution to be used for the experiments. For the actual experiments, the chamber was completely filled with the appropriate standard solution, through the luer attachments, taking care to avoid the formation of bubbles. Once the chamber was filled, it was mounted on the rotational stage within an enclosure whose temperature was set to $22^{\circ}C$ using a temperature control unit (AirTherm SMT, World Precision Instruments). During this time, the fluid suspension containing the objects or organisms to be tracked was also stored within this enclosure. After this a fluid mixing protocol was activated to achieve thermal equilibrium between the chamber and fluid. Such a thermal equilibration was necessary in order to prevent thermally driven flows from occurring in the chamber during the experiments. This mixing protocol consisted of a rotational motion of the chamber and periodically reversing the rotation direction. These motions leads to a shear-enhanced mixing of fluid in the chamber, and a correspondingly more effective heat transfer, both between azimuthally separated fluid parcels, as well as between the fluid and the chamber. This mixing protocol was carried out for 10 minutes prior to introducing the objects to be tracked. After this protocol the average background fluid motion when the fluidic chamber was at rest was measured using Particle-Image-Velocimetry (PIV), and was found to typically be $< 20\mu ms^{-1}$ (Fig. S5). Once thermal equilibrium was achieved, the objects or organisms to be tracked were introduced and the above equilibration procedure was again run for 10 mins to evenly suspend the objects or organisms in the fluid. Once this procedure was completed tracking could be started. This was achieved by manually locating an object of interest and then starting the automated tracker (Movie 1). Care was also taken to ensure significant air-circulation in the experimental enclosure and prevent sources of heat from being present near the fluidic chamber as these can cause thermally driven flows

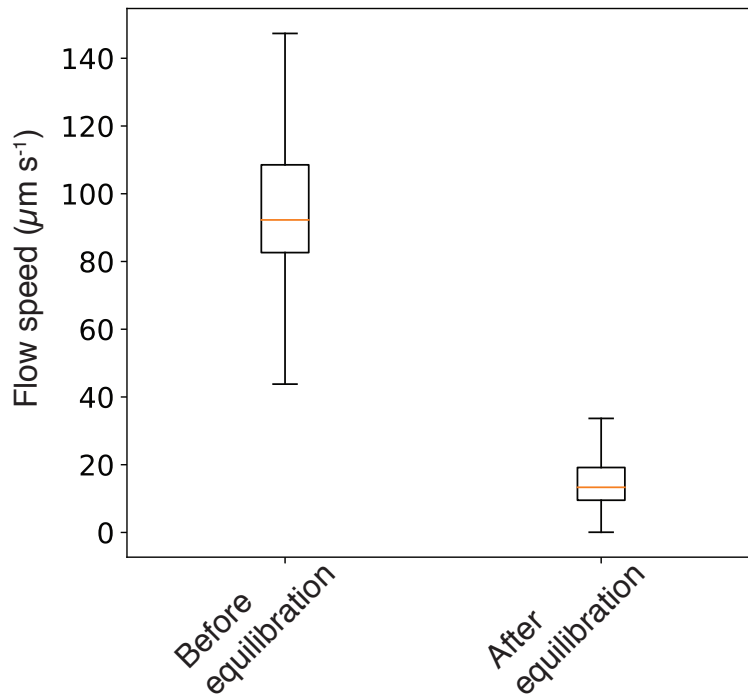


Figure S5 Background flow measurement. Background flow speeds in the circular fluidic chamber before and after the thermal equilibration procedure. This flow was measured over the microscope FOV for a duration of 6 seconds at 30 Hz using Particle Image Velocimetry. The box-plot is from the lower and upper quartile of the data, the line represents the median and whiskers show the range. Typical flow speeds were measured to be $< 20 \mu m s^{-1}$ after the thermal equilibration procedure.

114 to occur during long-term imaging.

115 **1.6 Abiotic experiments**

116 For validating our experiments we used density marker beads of known size and density
 117 purchased from Cospheric (Table S2). These beads have a precisely calibrated density which
 118 is slightly higher than water, as well as a mono-disperse size range (Table S2)). We measured
 119 the sedimentation velocity of these beads as a means to validate our tracking method and
 120 microscope. To perform this calibration, these beads were tracked in two ways. As a control,
 121 Eulerian tracks of the beads were obtained by allowing them to sediment down a vertical
 122 cuvette with a height of 150 mm and cross-sectional dimensions 15 mm × 3 mm. The beads

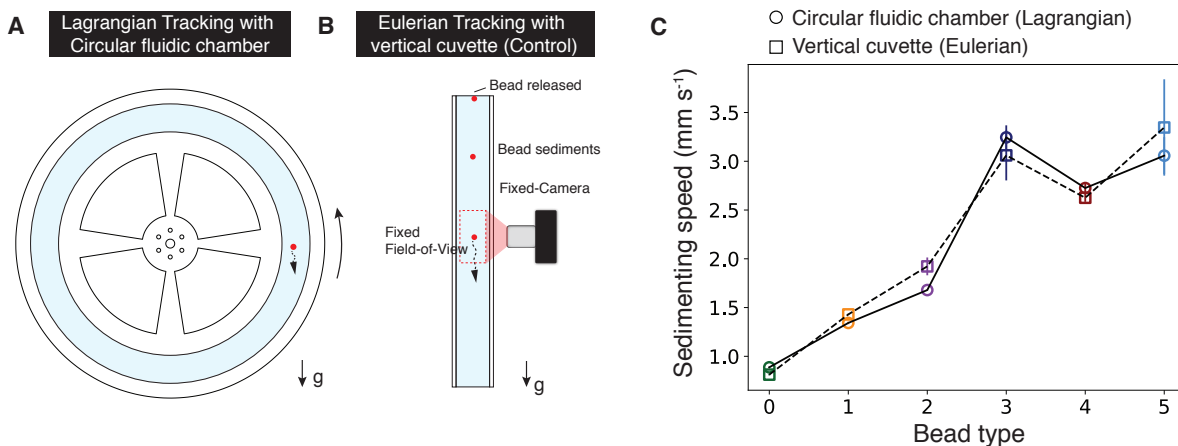


Figure S6 Validation of the vertical tracking method. We measured the sedimentation speeds of $250 \mu\text{m}$ density calibration beads using **(A)** the method presented in this work and **(B)** conventional fixed-camera method with a vertical cuvette. **(C)** Comparison of measured sedimentation speeds for 6 types of beads with different densities and diameters (data is for $n = 10$ tracks per bead type and tracking method) (see Table S2) using the vertical tracking method presented in this work (circles) and conventional method (squares). Error bars represent one standard deviation.

123 were imaged as they sedimented past a fixed camera mounted to image in the vertical plane
 124 at a location at the middle of the cuvette (see schematic in Fig. S6B), so as to avoid end
 125 effects. Lagrangian tracks of the density marker beads were obtained by tracking them using
 126 the circular fluidic chamber and tracking methodology developed in this work (Fig. S6A).
 127 Each bead was tracked for a 1 *min* after which the track was stopped and a new bead
 128 tracked. The sedimenting velocity, for both the tracking methods above, was obtained by
 129 a linear fit of measured time-traces of vertical displacement. The measured sedimentation
 130 velocities ($n = 10$ tracks, per bead, per method) are shown in Fig. S6C, and were found to
 131 be in good agreement.

132 The spherical beads used for measuring interactions between sedimenting particles were
 133 $500 \mu\text{m}$ solid glass spheres with density 2200 kgm^{-3} (Cospheric LLC., USA). The rods used
 134 for the experiments were pencil leads of different lengths in the range $0.5 - 2 \text{ mm}$, diameter
 135 $500 \mu\text{m}$ and density (2300 kgm^{-3}). The crystals used for the sedimentation-dissolution ex-
 136 periments were raw sugar crystals (Turbinado cane sugar) chosen since they dissolve slower

137 compared to refined sugar crystals of the same size. Marine detritus particles used in ex-
138 periments were collected during night-time (10 PM local time on 31 August 2018) plankton
139 tows off the coast of Monterey, California, USA using plankton net size of 100 μm and iso-
140 lated under a dissection scope. It was then suspended in filtered sea water from the Hopkins
141 marine station before tracking on the microscope.

142 **1.7 Marine invertebrate larvae experiments**

143 The larvae used for experiments were obtained by fertilizing adult animals collected off the
144 coast of Monterey, California, USA. The culturing procedure for larvae matched standard
145 protocols in the field [5]. Before experiments the larvae were transferred to filtered sea
146 water and allowed to acclimatize for an hour. Larvae were transferred to the circular fluidic
147 chamber using tubing at least three times their body size using gentle suction.

148 **1.8 Environmental patterning experiments with Volvox**

149 *Volvox aureus* colonies were obtained from Carolina Inc. and cultured in Volvox medium
150 (UTEX). For the environmental patterning experiments, we used a white LED array (Adafruit)
151 (Fig. S1A; Fig. S12) mounted at the ceiling of our experimental enclosure, which provided
152 a uniform top illumination at the sample location at the 3 o'clock location of the circular
153 fluidic chamber. The intensity of the LEDs was controlled using a Pulse-Width-Modulation
154 (PWM) signal from the Arduino microcontroller, in turn controlled by the desktop CPU
155 based on the virtual depth of the tracked organism (see Fig. S2A). Using this system any
156 temporal (and hence 'virtual-depth'-based) intensity profile could be programmed into our
157 experiments as a function of the virtual depth of the organism being tracked. In our exper-
158 iments we simulated an artificial profile which alternated between light and dark for every
159 20 mm in height gained by the organism. Red (625 nm , Fig. S12) LEDs were used to image
160 the organisms since this is a wavelength to which Volvox is insensitive [6].

161 **1.9 Single cell tracking experiments**

162 Pyrocystis noctiluca were obtained from UTEX (UTEX LB 2504) and cultured in F/2
163 medium (Bigelow Labs) at 20-22°C while maintaining a 12/12H light/dark cycle. Cells
164 were loaded into the chamber 3H after the beginning of the light cycle and allowed to equili-
165 brate for 1H with gentle rotation in the apparatus before tracking. Cells were imaged using
166 red (625nm) illumination under a white LED array maintaining the daylight cycle. 3 μ m
167 latex beads were included for PIV measurements.

168 **1.10 Data analysis procedures**

169 **1.10.1 Flow field calculation using Particle Image Velocimetry (PIV)**

170 For calculating flow fields around objects and freely swimming organisms, the fluid was
171 seeded with neutral density tracer particles (2 or 3 μ m polystyrene beads, Polysciences)
172 with a typical final concentration of 0.05% by volume of beads in the standard solution
173 being used in the particular experiment. For resolving dynamic flow fields a sampling rate
174 of at least 30 Hz was used, and this was adjusted to a higher value based on the flow speeds
175 in different experiments. The images obtained were analyzed using an open-source PIV
176 software implemented on Python [7]. The parameters used for the PIV data analysis are
177 provided in Table S3. The data obtained from the PIV analysis was post-processed as follows.
178 Outlier vectors were detected using the signal-to-noise ratio threshold of the correlation peaks
179 heights, and replaced by the local nearest neighbour average. The data was also interpolated
180 on to a 2x finer grid than that used for calculating the correlations for estimating flows near
181 the surface of organisms. For calculating path-lines of tracer particles in the fluid we used
182 a sliding-window-based maximum intensity projection method as implemented using the
183 Flowtrace software package [8].

1.11 Mapping measured angular displacement of the chamber to virtual depth of object

Our tracking method uses a circular chamber as a 'hydrodynamic treadmill' to track vertical motion over unlimited scale. This method implies that the tracked object does not move relative to the lab reference frame, and its displacement with respect to the fluid is measured by the angular displacement of the chamber. We can map this angular displacement to the 'virtual depth' of the object as follows:

$$Z^{i+1} = Z^i + (Z_{com}^i - Z_{com}^{i-1}) + (R_{center} + X^i)(\theta^i - \theta^{i-1}), \quad (1)$$

where the superscripts i denote discrete time in control-loop cycles, so that Z^i is the 'virtual depth' of the object at time i , Z_{com}^i is the centroid of the object relative to the optical Field-of-View (FOV), R_{center} is the radius of the chamber's centerline, X^i is the displacement along the X -axis, as measured from the chamber center-line, and θ^i is the angular displacement of the circular chamber.

The displacement along the x direction is give by:

$$X^i = X_{FOV}^i + X_{com}^i, \quad (2)$$

$$Y^i = Y_{FOV}^i, \quad (3)$$

where X_{FOV}^i is the displacement of the optical FOV relative to the center-line of the chamber and X_{com}^i is the x displacement of the object's centroid compared to the optical FOV. Note that our y displacement is taken as the location of the focal plane of the optical system, which tracks objects movements using our focus tracking system (see §1.4.1). Also note that the FOV along the x and y directions, unlike z , are not fixed in the lab reference frame, which is why the update formulae in Eqs. (2) and (3) are different from those in Eq. (1).

2 Supplementary Text

2.1 Calculation of relevant non-dimensional numbers for tracking using a circular fluidic chamber

To understand the design space and operating parameters involved in using a circular fluidic chamber as a “hydrodynamic treadmill” to track vertical motion, we performed a scaling analysis of the various physical effects at play. This initial scaling analysis helps us understand which of these physical effects are important, which then motivated us to do a more detailed analysis of those effects on the tracking performance, presented in subsequent sections and also discussed in the main text of this work.

Consider the tracking of objects with a size scale d , a vertical speed relative to the ambient fluid of u_{obj} , mean density ρ_{obj} , in an ambient fluid with density ρ_f and kinematic viscosity ν . In our work, we restrict ourselves to objects or organisms which are small, with sizes which are $\mathcal{O}(mm)$ and smaller. Also these objects have a typical speed relative to the fluid of a few body lengths per second. For the sake of this analysis, we consider a spherical object with diameter $d = 1\text{ mm}$, and speed $u_{obj} = 1\text{ mms}^{-1}$, immersed in water ($\nu \approx 10^{-6}\text{ m}^2\text{ s}^{-1}$). For such an object, the Reynolds number, which quantifies the relative importance of inertial and viscous effects in the flow [9], is:

$$Re = \frac{u_{obj}d}{\nu} \approx 1. \quad (4)$$

This sets an upper bound, at least for the biological organisms considered in this work.

A more relevant dimensionless number for tracking, is the Stokes number which quantifies the relative time scales of the object and the time scale of the flow [9], and is given by :

$$Stk = \frac{t_{obj}}{t_{flow}}, \quad (5)$$

where t_{obj} is the relaxation time-scale of the object, i.e. the time scale over which the object’s

224 velocity decays exponentially due to fluid drag, and t_{flow} is a characteristic time-scale of the
 225 flow. In our tracking system, the relevant time-scale for the flow is that of viscous diffusion
 226 of momentum since this sets the time over which the fluid changes velocity due to changes in
 227 the chamber’s velocity. This time scale is given by W^2/ν , where W is smallest cross-sectional
 228 chamber dimension (the chamber width). The relevant relaxation time-scale for the object,
 229 for the case of Stokes flow ($Re < 1$) is given by:

$$t_{obj} = \frac{\rho_{obj}d^2}{18\mu}, \quad (6)$$

230 where μ is the dynamic viscosity of the fluid. For most biological organisms their mean
 231 density falls in the range of a 5 – 10% excess density to that of water [10]. Assuming these
 232 density ranges and the parameters used above for the size and speed, the Stokes number
 233 number is $Stk \approx 0.006$. Since $Stk \ll 1$, the object’s inertial time-scale is negligible, and the
 234 object is advected by the ambient fluid motion. This means that the net motion relative to
 235 the fluid is, at leading order, only because of a body forces, such as those due to gravity,
 236 and/or active swimming stresses. Smaller, higher order Faxén’s corrections exist due to the
 237 finite size of the object in a transient non-uniform flow profile [11], which we neglect.

238 2.1.1 Effects of chamber curvature and rotation

239 Our next considerations are understanding the role of curvature of the chamber on the
 240 tracked object’s motion. Firstly, since the tracked object is immersed in a fluid that is
 241 globally undergoing a solid-body-rotation, it is subject to an angular velocity given by:

$$\Omega_{rotation} = u_{obj}/R, \quad (7)$$

242 where R is the object’s radial location. Using typical values of $u_{obj} = 1 \text{ mm s}^{-1}$ and
 243 $R = 100 \text{ mm}$, we see that $\Omega_{rotation} = 10^{-2} \text{ s}^{-1}$. We will see in subsequent sections, that this
 244 is a negligible contribution to the object’s orientation dynamics, and is sub-dominant to the

245 effects of small bottom-heavy density distributions, as well as object shape, both of which
 246 control the object's orientations at much faster time-scales.

247 Chamber rotation gives rise to centrifugal forces which establish a radial pressure gradi-
 248 ent, in addition to the hydrostatic gradient due to the vertical orientation of the chamber.
 249 The centrifugal contribution to this pressure gradient is given by:

$$\left. \frac{\partial p}{\partial r} \right|_{centrifugal} = \frac{\rho_f u_{obj}^2}{R}, \quad (8)$$

250 where R is the radial location of the object relative to the center of the chamber.

251 This radial pressure gradient causes a radial drift of objects embedded in the fluid which
 252 can be computed considering the balance of buoyant and fluid drag forces [9]:

$$U_{centrifugal} = \frac{(\rho_s - \rho_f)V_{obj}u_{obj}^2}{C_D\mu dR}, \quad (9)$$

253 where V_{obj} is the object's volume and C_D is the drag coefficient. For a spherical object, this
 254 simplifies to the expression, rewritten as a relative velocity ratio:

$$\frac{U_{centrifugal}}{u_{obj}} = \frac{(\rho_s - \rho_f)d^2u_{obj}}{18\mu R}, \quad (10)$$

255 which for the parameters used above, and $\rho_{obj} = 1100\text{kgm}^{-3}$ is $U_{centrifugal}/u_{obj} = 6.35 \times 10^{-5}$.
 256 Thus drift due to centrifugal forces can be safely neglected.

257 **2.2 Hydrodynamic considerations for tracking**

258 Tracking vertical motion using a circular fluidic chamber with a contiguous annulus of fluid
 259 (i.e. with no walls normal to the direction of chamber rotation) implies that the fluid has a
 260 separate degree-of-freedom and does not rigidly move with the chamber walls. Any change
 261 in momentum of the chamber is transmitted to the fluid only via viscous diffusion with
 262 an associated temporal delay and spatial non-uniformity in the fluid motion. To achieve
 263 effective tracking, we need to account for this fluid motion and understand its effects on

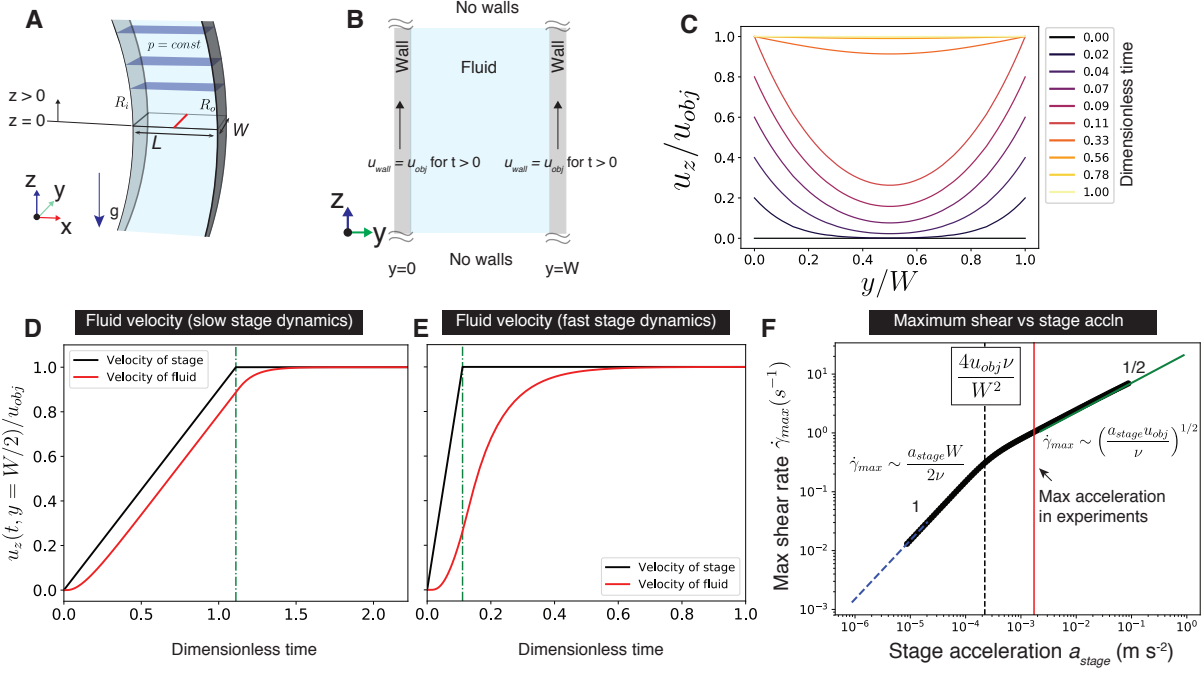


Figure S7 Hydrodynamic considerations for vertical tracking using a “hydrodynamic treadmill”. **(A)** Coordinate system, also showing iso-bars when the fluid is at rest. **(B)** Geometry and boundary conditions for solving for the flow due to impulsive start-up motion of the channel walls. **(C)** Theoretically predicted flow velocity as a function of distance across the channel for an impulsive start-up motion of the channel walls (colored contours: time series of flow velocity profiles). **(D)-(E)** Flow velocity vs dimensionless time ($\tilde{t} = t\nu/W^2$) at the center-line of the channel for the limits of (D) small and (E) large stage accelerations. **(F)** The spatio-temporal maximum in shear rate developed in the fluid during a start-up motion with finite stage acceleration a_{stage} . The shear rate has two asymptotic regimes based on the magnitude of the stage acceleration. For slow stage dynamics, compared to the viscous time scale ($\tau_{\text{stage}} \gg \tau_{\text{visc}}$), the shear rate scales as $\dot{\gamma}_{\text{max}} \sim a_{\text{stage}} W / (2\nu)$ (blue dashed line), where $\tau_{\text{stage}} = u_{\text{obj}} / a_{\text{stage}}$ and $\tau_{\text{visc}} = W^2 / \nu$. For fast stage dynamics ($\tau_{\text{stage}} \ll \tau_{\text{visc}}$), the shear rate scales as $\dot{\gamma}_{\text{max}} \sim (a_{\text{stage}} u_{\text{obj}} / \nu)^{1/2}$ (green solid line). The cross-over between the two scaling regimes is given by solving $\tau_{\text{stage}} \sim \tau_{\text{visc}}$. The shear-rate corresponding to the maximum stage acceleration (red vertical line) used in our experiments is $\approx 1 \text{ s}^{-2}$.

264 the tracks obtained. Towards this, we model the dynamics of fluid motion in response to
 265 arbitrary control inputs to the chamber.

266 The fluidic chamber is an annulus with inner and outer radii R_i and R_o , respectively.
 267 The annulus has a rectangular cross-section $L \times W$, where $L = R_o - R_i$ is the length of the
 268 chamber along the plane of the annulus and W is the chamber width along the optical axis
 269 (Fig. S7A). Since the cross-section is long and thin i.e. $L \gg W$, the fluid locally responds
 270 to the nearest walls, and therefore it is sufficient to consider the fluid motion at the center
 271 of the annulus $R = (R_i + R_o)/2$, along a one dimensional section along the smallest chamber
 272 dimension, which is the width W (see Fig. S7A). Also since $R_i, R_o \gg W$, we neglect the
 273 effects of the chamber curvature at leading order. At leading order, the effects of gravity
 274 also do not contribute to fluid motion and only result in a hydrostatic gradient given by:

$$p_{static}(z) = p_{chamber} + (R_o - z)\rho_f g, \quad (11)$$

275 where $p_{chamber}$ is the ambient pressure in the chamber, and the height z is measured from
 276 the horizontal plane at the 3 O'clock azimuthal location of the circular chamber (Fig. S7A).
 277 With these assumptions, the problem for the fluid motion reduces to that of start-up flow
 278 in a channel of width W , where the fluid motion is purely due to the motion of the channel
 279 walls (Fig. S7B). For convenience, we use a coordinate system where the channel walls are
 280 at $y = 0, W$ (Fig. S7B). Since the chamber is circular, the problem reduces to one where the
 281 channel is infinite in extent along the length, therefore making the problem one dimensional
 282 (Fig. S7B).

283 We now solve for the fluid motion in this configuration for a start-up motion of the
 284 walls. With the above assumptions, the flow is uni-directional so that $\mathbf{u} = u_z(t, y)\mathbf{e}_z$ and
 285 the dynamic pressure field can be written as $p_{dynamic} = p(t, y)$. The fluid flow equations,
 286 therefore, reduce to a diffusion equation for z -momentum [9] which is given by:

$$\frac{\partial u_z}{\partial t} = \nu \frac{\partial^2 u_z}{\partial y^2}, \quad (12)$$

287 with the following initial and boundary conditions:

$$\begin{cases} u_z(t=0, y) = u_0(y) = 0, & \text{initial conditions} \\ u_z(t, 0) = u_z(t, W) = U, & \text{boundary conditions.} \end{cases} \quad (13)$$

288 Here U is the velocity of the walls.

289 Solution of this equation is standard [9], and can be solved by decomposing the solution
 290 to a general and particular solution, where the general solution satisfies a homogeneous
 291 boundary condition. A standard separation of variables type approach results in the solution
 292 for the fluid velocity:

$$u_z(t, y) = U \left[1 - \frac{4}{\pi} \sum_{n=0}^{\infty} \frac{1}{(2n+1)} \exp\left(-\frac{(2n+1)^2 \pi^2}{W^2} \nu t\right) \sin\left(\frac{(2n+1)\pi y}{W}\right) \right], \quad (14)$$

293 and the solution for the pressure is $p_{dynamic} = 0$.

294 This constitutes the response of the fluid to a step change in velocity of amplitude U .
 295 Thus one can write down the step response of the fluid to a unitary step function in wall
 296 velocity, or equivalently, the impulse response of the fluid to a delta distribution in wall
 297 acceleration, as follows:

$$u_{\delta}(t, y) = \left[1 - \frac{4}{\pi} \sum_{n=0}^{\infty} \frac{1}{(2n+1)} \exp\left(-\frac{(2n+1)^2 \pi^2}{W^2} \nu t\right) \sin\left(\frac{(2n+1)\pi y}{W}\right) \right]. \quad (15)$$

298 Having derived the impulse response of the fluid, we can now derive the response to a general
 299 velocity profile of the walls. We use the fact that the governing equations for the fluid motion
 300 (Eqs. (12), (13)) constitute a linear, time-invariant (LTI) dynamical system, that is, the fluid
 301 velocity can be written as:

$$u_z(t, y) = \mathcal{F}[a(t)] \quad (16)$$

where $a(t)$ is the acceleration profile of the walls, and \mathcal{F} is a LTI operator. Since \mathcal{F} is LTI it obeys the convolution theorem, so that we have:

$$u_z(t, y) = u_z(t = 0, y) + \int_0^t a_{wall}(\tau) u_\delta(t - \tau) d\tau \quad (17)$$

where u_δ is the impulse response derived in Eq. (15). Using this we can derive the fluid velocity profile for general motion of the walls, as shown in Fig. S7D, for the case of a constant acceleration to a maximum velocity. As seen from Eq. (17), this response depends on the past history of wall motion via a kernel that decays over the viscous time scale W^2/ν .

Using the Eq. (17), We find, as expected, that the maximum shear rate always occurs at the walls ($y = 0, W$). Further, we calculate the time dynamics of the shear rate developed in the fluid, which is marked by an initial growth to temporal maximum value followed by a decay to zero, as the fluid velocity becomes a solid body motion with the walls. It is this spatio-temporal maximum in shear rate, that we use in our characterization of the effects of shear on the orientation of tracked objects, discussed next. Since this maximum shear rate occurs at the walls and is also a temporal maximum, it sets the upper bound for the shear rate experienced by a tracked object anywhere in the channel, at any time during its track. Using the relevant value for the maximum stage acceleration used in our experiments, we find that this maximum in shear rate does not exceed about $1s^{-1}$, at any point of our tracing.

2.3 Effects of stage and fluid response time on tracking

We now consider the effects of stage and fluid response times on vertical tracking. The fluid response time has been derived in §2.2, and has a viscous lag which scales as $\tau_{visc} \sim W^2/\nu$. The stage response time is determined by the specific motion profile implemented on our

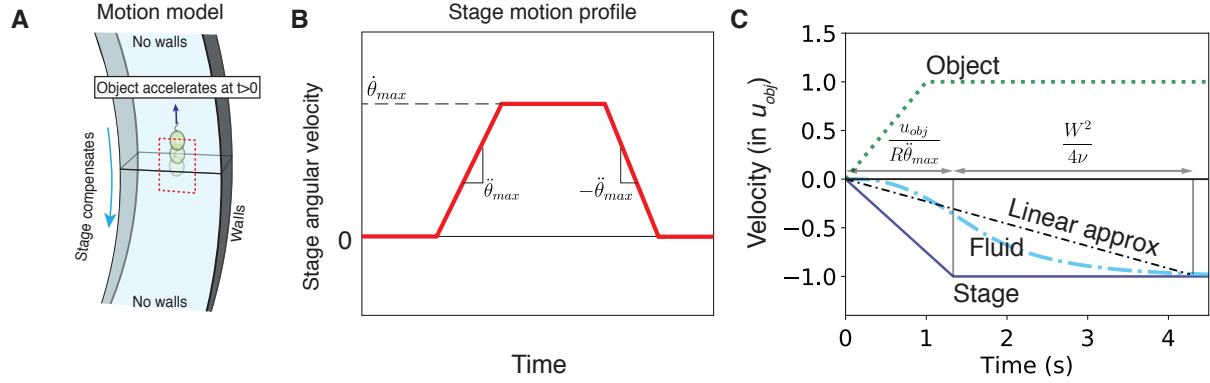


Figure S8 Effects of stage and fluid response time on tracking. **(A)** Motion model for an organism or object that changes its vertical velocity by u_{obj} . To track this motion, the stage compensates by accelerating in the opposite direction at a rate $\dot{\theta}_{max}$. **(B)** Motion profile implemented on our rotational stage. A change in position, is implemented by acceleration at a constant rate $\dot{\theta}_{max}$ to a maximum speed $\dot{\theta}_{max}$ and deceleration at a constant rate $-\dot{\theta}_{max}$. This angular velocity profile maps to a translational velocity of the walls at the object's radial location as $a_{stage} = R\ddot{\theta}(t)$. **(C)** Translational velocity vs time of the object (green dotted curve), stage (solid blue curve) and fluid (cyan dash-dotted curve). The fluid velocity lags behind that of the stage due to the viscous delay of $\tau_{visc} \sim W^2/\nu$ in the transfer of momentum. For analytical calculations, this delay can be modelled, without worrying about the actual details of the fluid-velocity time-series (cyan dash-dotted curve) using a linear approximation (black dash-dotted curve), as shown.

323 rotational stage. This motion profile consists of acceleration at a rate $\ddot{\theta}_{max}$ to a maximum
 324 angular velocity of $\dot{\theta}_{max}$, and is given in Fig. S8 B.

325 We consider a motion model for a tracked object, at the channel center-line, wherein it
 326 accelerates and changes its vertical velocity by u_{obj} at an acceleration a_{obj} , and the stage
 327 compensates by moving in the opposite direction at an acceleration $a_{stage} = R\ddot{\theta}_{max}$, where R
 328 is the instantaneous radial location of the object (Fig. S8A). The object's velocity relative
 329 to the fluid is:

$$u_{obj|fluid}(t) = \begin{cases} a_{obj}t, & t \leq u_{obj}/a_{obj} \\ u_{obj}, & t > u_{obj}/a_{obj} \end{cases} \quad (18)$$

330 The stage's velocity relative to the lab is:

$$u_{stage|lab}(t) = \begin{cases} -a_{stage}t, & t \leq u_{obj}/a_{stage} \\ -u_{obj}, & t > u_{obj}/a_{stage}. \end{cases} \quad (19)$$

331 While we have numerically derived the fluid's velocity profile in §2.2, in order to make
 332 analytical progress towards deriving an expression for the tracking error as a function of
 333 time, we make a simplifying assumption for the velocity time dynamics of the fluid. We
 334 make a linear approximation for the fluid velocity such that the fluid accelerates at a constant
 335 rate, but at a rate slower than the stage (Fig. S8C). Therefore while the stage takes a time
 336 $\tau_{stage} = u_{obj}/(R\ddot{\theta}_{max})$ to accelerate to the object's velocity, the fluid at the channel center-line
 337 takes a time given by $\tau_{stage} + \tau_{visc} = u_{obj}/(R\ddot{\theta}_{max}) + W^2/4\nu$, where $W^2/4\nu$ is the time-scale for
 338 momentum to diffuse from the channel walls to its center-line. Therefore, the fluid velocity
 339 at the center-line can be approximated as:

$$u_{fluid|lab}(t) = \begin{cases} -\frac{u_{obj}}{\tau_{stage} + \tau_{visc}}t, & t \leq \tau_{stage} + \tau_{visc} \\ -u_{obj}, & t > \tau_{stage} + \tau_{visc} \end{cases} \quad (20)$$

340 Since the Reynolds and Stokes numbers are small for the objects we consider (see §2.1),
 341 both fluid and object inertia is negligible, and the object velocity relaxes to that set by
 342 the instantaneous fluid drag. Based on this, the object's velocity relative to the lab (or
 343 microscope's FOV) is given by:

$$u_{obj|lab}(t) = \begin{cases} a_{obj}t - \frac{u_{obj}}{\tau_{stage} + \tau_{visc}}t, & t \leq u_{obj}/a_{obj} \\ u_{obj} - \frac{u_{obj}}{\tau_{stage} + \tau_{visc}}t, & u_{obj}/a_{obj} < t \leq \tau_{stage} + \tau_{visc} \\ 0, & t > \tau_{stage} + \tau_{visc}. \end{cases} \quad (21)$$

344 Note that we have made the assumption above that $u_{obj}/a_{obj} < \tau_{stage} + \tau_{visc}$, which is the
 345 relevant limit to consider since we are interested in deriving the limits of our tracking method
 346 for fast object dynamics. Using this we get the vertical tracking error (displacement of the

347 object relative to the microscope FOV) as:

$$z_{error}(T) = \int_0^T u_{obj|lab}(t) dt. \quad (22)$$

348 Note that it is sufficient to consider $T < \tau_{stage} + \tau_{visc}$, since the velocity at later times is zero.

349 Using Eq. (21), we can write Eq. (22) as:

$$z_{error}(T) = \int_0^{u_{obj}/a_{obj}} \left(a_{obj} - \frac{u_{obj}}{\tau_{stage} + \tau_{visc}} \right) t dt + \int_{u_{obj}/a_{obj}}^T \left(u_{obj} - \frac{u_{obj}}{\tau_{stage} + \tau_{visc}} t \right) dt. \quad (23)$$

350 To obtain a tracking condition, we require that the vertical tracking error for an object
 351 starting at the center of the FOV not cross the FOV, which can be written as the inequality:

$$z_{error}(T) \leq L_{FOV}/2, \quad (24)$$

352 where L_{FOV} is the vertical extent of the microscope's FOV. Substituting Eq. (23) in Eq.
 353 (24), integrating and simplifying, we obtain the following inequality:

$$T^2 - 2(\tau_{stage} + \tau_{visc})T + (\tau_{obj} + L_{FOV}/u_{obj})(\tau_{stage} + \tau_{visc}) \geq 0. \quad (25)$$

354 To derive the tracking condition, one can use the equality condition for Eq. (25), since
 355 that denotes the point when the object reaches the edge of the FOV. Thus the condition
 356 for tracking success at all times requires that equation $T^2 - 2(\tau_{stage} + \tau_{visc})T + (\tau_{obj} +$
 357 $L_{FOV}/u_{obj})(\tau_{stage} + \tau_{visc}) = 0$, have no real roots. This gives us the condition for tracking
 358 success as:

$$\tau_{visc} + \tau_{stage} \leq \frac{L_{FOV}}{u_{obj}} + \tau_{obj} \quad (26)$$

2.4 Effects of transient shear on object orientation

We now consider the effects of transient shear on the orientation of the tracked objects. This shear is generated since changes to the rotational velocity of the fluidic chamber leads to a transient non-uniform flow profile, which on the scale of a microscale object is a transient shear flow (Fig. S9 A). Thus in the limit $d \ll W$, where d is the object size, the flow is locally given by $\mathbf{u} = -\dot{\gamma}y\hat{\mathbf{e}}_z$, where $\dot{\gamma}$ is the shear rate and $\hat{\mathbf{e}}_z$ is the unit vector along the z-direction (upward direction). This flow can be decomposed into an extensional and vortical component, where the extensional tensor is given by:

$$\mathbf{E} = \begin{bmatrix} 0 & 0 & 0 \\ 0 & 0 & -\dot{\gamma}/2 \\ 0 & -\dot{\gamma}/2 & 0 \end{bmatrix} \quad (27)$$

and the vorticity is $\boldsymbol{\omega} = \dot{\gamma}\hat{\mathbf{e}}_x$.

We model the tracked organism as a prolate spheroid with aspect ratio q , with a bottom-heavy density distribution which is parameterized by a separation distance Δd between the center-of-buoyancy and center-of-mass. The orientation of such a spheroidal, bottom-heavy object can be written using Jeffery's theory [12, 13, 14] as:

$$\dot{\mathbf{p}} = \frac{1}{2B}[\hat{\mathbf{e}}_z - (\hat{\mathbf{e}}_z \cdot \mathbf{p})\mathbf{p}] + \frac{1}{2}\boldsymbol{\omega} \wedge \mathbf{p} + \beta[\mathbf{E} \cdot \mathbf{p} - (\mathbf{E} : \mathbf{p}\mathbf{p})\mathbf{p}], \quad (28)$$

where $B = \mu\alpha_{\perp}/(2g\rho_{obj}\Delta d)$, is a time-scale for reorientation due to gravity, μ is the dynamic viscosity of the fluid, α_{\perp} is a drag coefficient for rotation of a prolate spheroid [15], ρ_{obj} is the object's mean density, and $\beta = (1 - q^2)/(1 + q^2)$.

Substituting the expressions for the extension tensor and vorticity in Eq. (28), and further simplifying for the case of small angles θ , we have, to leading order in θ :

$$\dot{\theta} = -\frac{\theta}{2B} + \frac{\dot{\gamma}(1 - \beta)}{2}. \quad (29)$$

377 The first of the terms on the right-hand-side of Eq. (29) represents the orientation stabilizing,
 378 gravitactic term, while the second is the destabilizing term due to the shear. Each of these
 379 terms is additionally the inverse of a time-scale. Thus, to quantify the relative strengths of
 380 the terms we can consider the ratio of the two time-scales, namely the gravitactic time-scale
 381 $\tau_{grav} = B$, and the shear time scale $\tau_{shear} = [\dot{\gamma}(1-\beta)]^{-1}$. The object's orientation is therefore
 382 stable and orientation perturbation due to shear, negligible when:

$$\boxed{\frac{\tau_{shear}}{\tau_{grav}} \gg 1} \quad (30)$$

383 For the sake of our analysis to determine the operating limits of vertical tracking, we further
 384 consider the spatio-temporal maximum in shear rate, so as to include a large safety margin.
 385

386 2.5 Point spread function of the optical system

387 Point spread function of the optical system is simulated by Zemax. That the object plane
 388 is in water and the presence of a 1.5 mm thick acrylic wall are taken into account (Figure
 389 S10). The root-mean-square (RMS) voltage applied to the liquid-lens is set to be 39.5V,
 390 which is the offset value used in experiments. Cross section of the point spread function and
 391 its maximum intensity projection to the y and z axes are plotted in Figure S10. Lateral and
 392 Axial FWHM are 2.8 μm and 116 μm respectively.

393 2.6 Liquid-lens chracterization

394 Relationship between the RMS voltage applied to the liquid-lens and the relative working
 395 distance is obtained by imaging a calibration slide mounted on a translation stage and
 396 recording the stage micrometer reading when the slide is adjusted to be in focus for different
 397 liquid-lens RMS voltages. Linear fit of the data yields a coefficient of 73.8 $\mu m/V_{rms}$ (Figure
 398 S10).

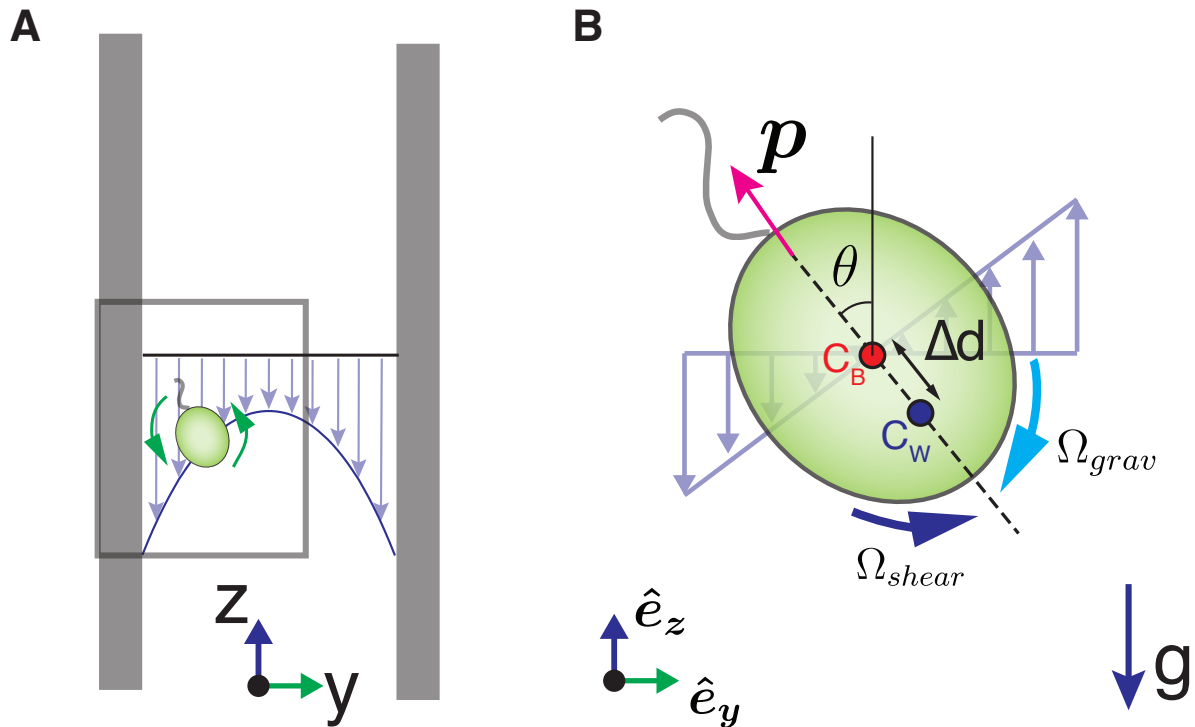


Figure S9 Effects of transient shear on object orientation. **(A)** Acceleration of the circular fluidic chamber causes a transient shear in the fluid which at the scale of a microscale object is a simple shear flow. **(B)** This simple shear flow can modify the object's natural orientation (p) which is set by a bottom-heavy density distribution that tends to align the orientation to the vertical. The bottom-heavy density distribution can be parametrized by a separation Δd between the center-of-buoyancy (C_B) and the center-of-mass (C_M).

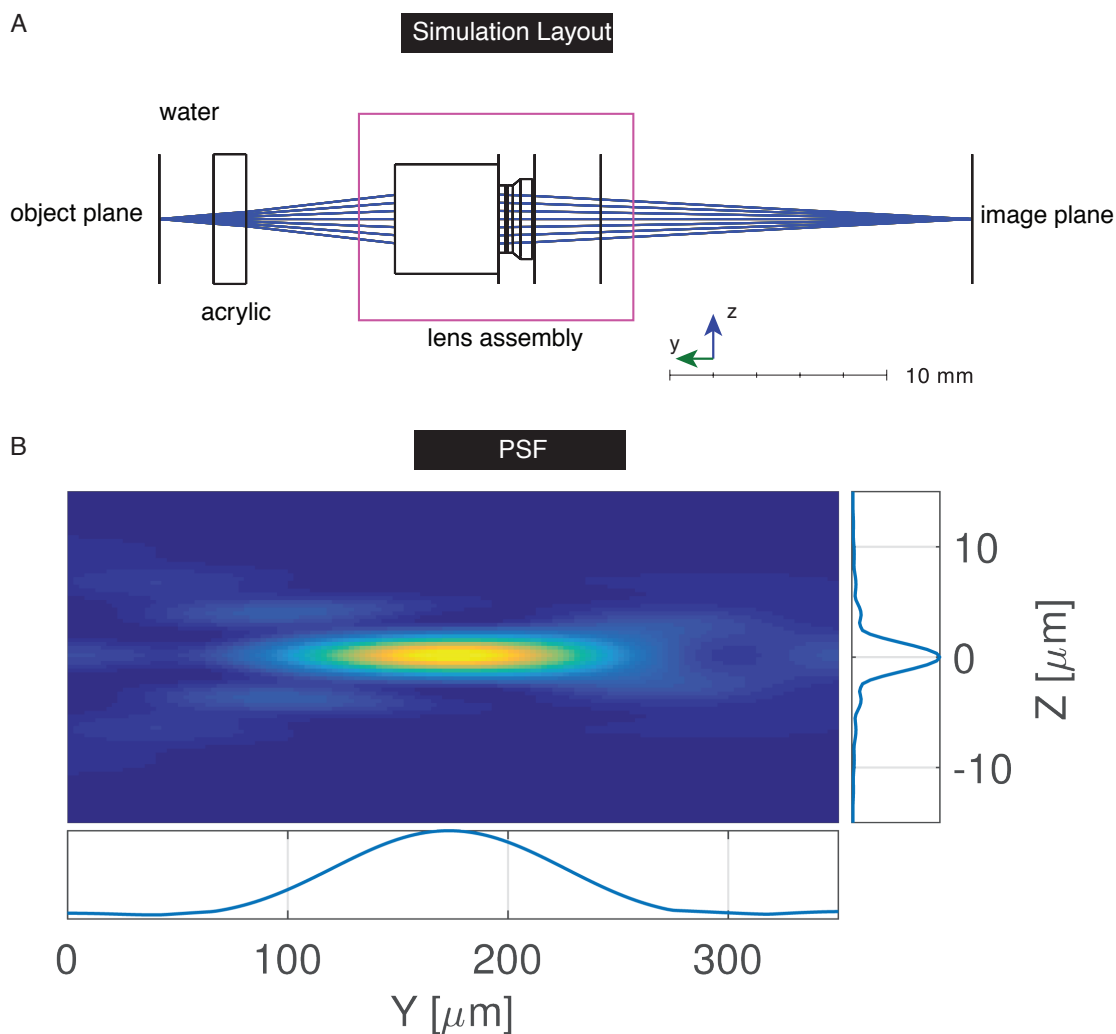


Figure S10 Point spread function of the optical system. **(A)** Simulation layout. The point spread function is simulated in Zemax. In the simulation a black box model of the liquid-lens assembly provided by the vendor (Corning) is used. The simulation takes into account the presence of acrylic chamber wall and the fluid (assumed to be fresh water with refractive index of 1.33). **(B)** Simulated point Spread Function. Lateral and Axial FWHM are $2.8 \mu\text{m}$ and $116 \mu\text{m}$ respectively.

399 Frequency response of the liquid-lens is obtained by converting the modulation of optical
400 power (inverse of the focal length) to modulation of beam size of a large diameter laser
401 beam that is focused by the liquid-lens in a fixed plane where the nominal beam size is a
402 few millimeters (Figure S11). A biased photodetector (DET036A, ThorLabs, load resistor
403 not shown) is placed such that part of the beam overlaps with the detector. The voltage
404 output, along with the sine waveform, generated using an Arduino’s built-in DAC, that
405 encodes the RMS voltage applied to the liquid-lens, are recorded by an oscilloscope. The
406 frequency of the modulation is swept and the amplitude and relative phase (between the
407 two recorded waveforms) are extracted by fitting sine functions to the recorded oscilloscope
408 traces. The obtained amplitude and phase response are shown in Figure S11. The phase
409 response measured here is used to generate a look-up table that applies a phase correction
410 when using the liquid-lens for our focus tracking method.

Larva name	Larva type	Days post fertilization
<i>S. californicum</i>	Tornaria	28
<i>D. excentricus</i>	Pluteus	11
<i>S. purpuratus</i>	Early Pluteus	2
<i>P. miniata</i>	Bipinnaria	12
<i>P. parvimensis</i>	Gastrula	2
<i>Owenia spp.</i>	Trochophore	4
<i>O. spiculata</i>	Pluteus	12
<i>C. fornicata</i>	Veliger	13

Table S1 Marine invertebrate larvae information.

Bead type	Color code	Size range (μm)	Density ($g\ cc^{-1}$)
0	Green	212 - 250	1.02 ± 0.005
1	Orange	212 - 250	1.04 ± 0.005
2	Violet	212 - 250	1.06 ± 0.005
3	Dark blue	250 - 300	1.08 ± 0.005
4	Red	212 - 250	1.09 ± 0.005
5	Blue	212 - 250	1.13 ± 0.005

Table S2 Density calibration beads (Cospheric) used for control experiments.

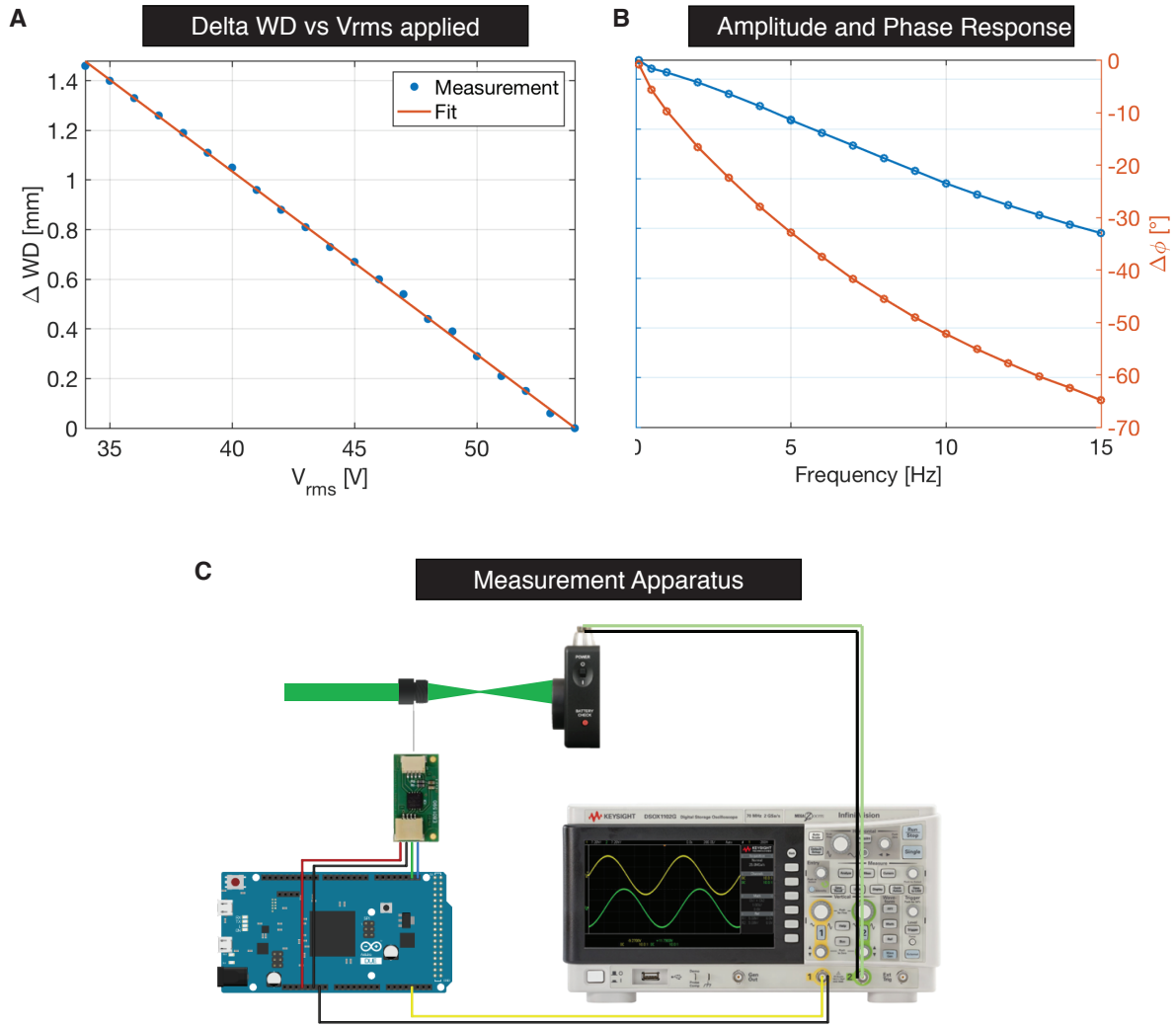


Figure S11 Liquid-lens Characterization. **(A)** Measured change in working distance vs voltage (root mean squared) applied to the liquid-lens. 20 V change in applied voltage (root mean squared) corresponds to 1.48 mm change in working distance, with coefficient of $73.8\mu\text{m}/V_{rms}$. **(B)** Amplitude and phase response of the liquid-lens vs modulation frequency. **(C)** Measurement apparatus for obtaining the amplitude and phase response.

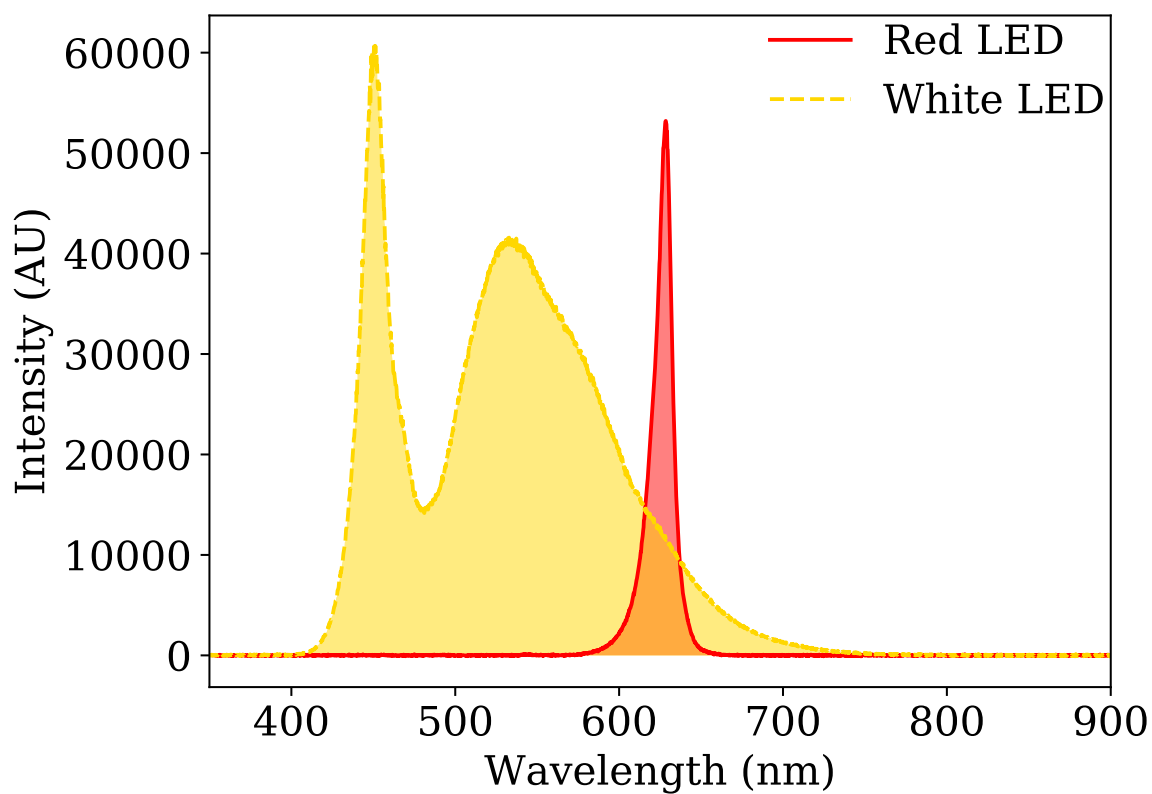


Figure S12 Measured intensity spectra for the two types of LED illumination used in experiments. The spectrum was measured using an Ocean Optics Spectrometer and a standard desktop computer.

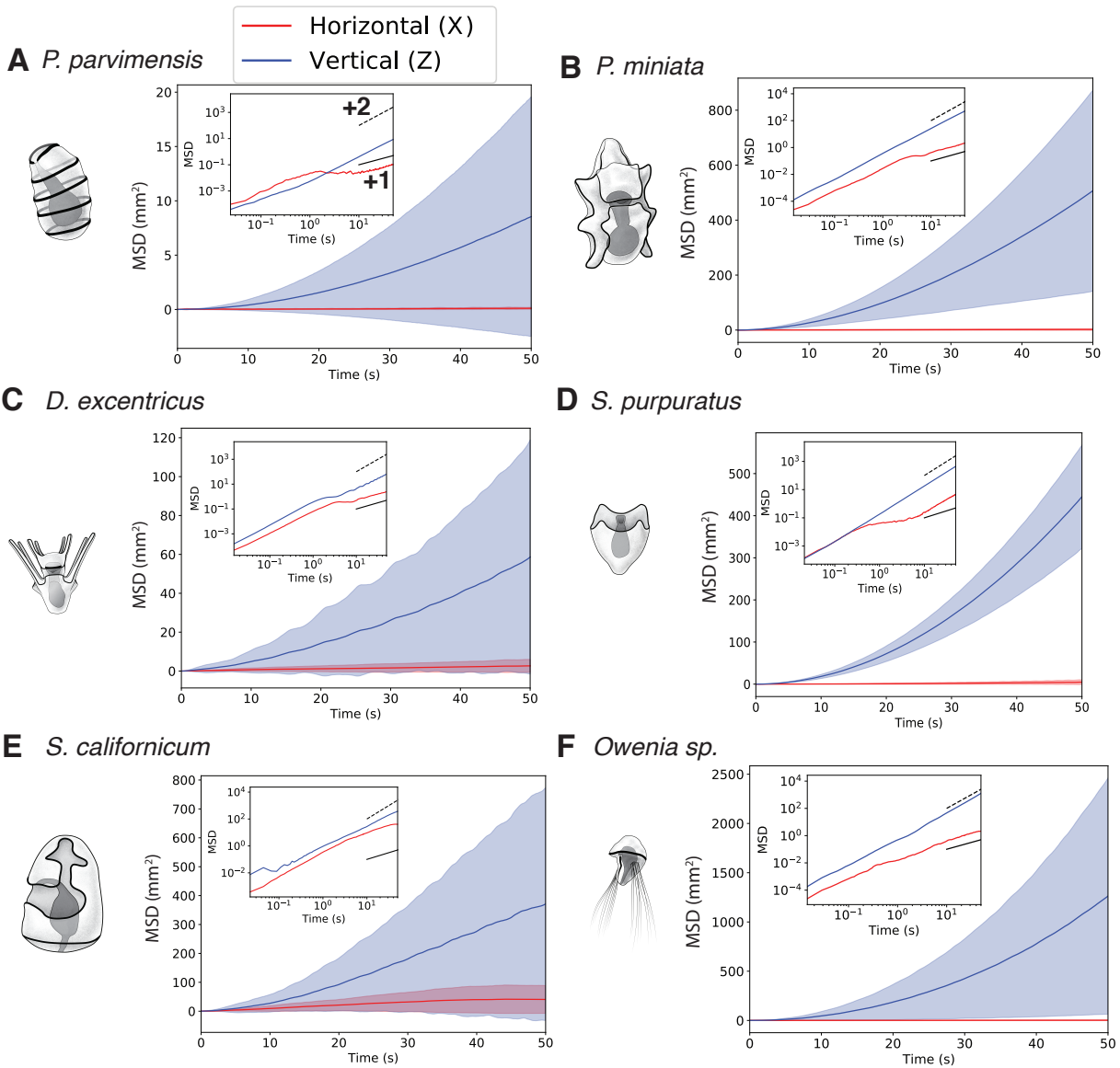


Figure S13 Measured vertical (z) and horizontal (x) Mean-Squared-Displacements (MSD) vs time for different marine invertebrate larvae. The insets show a log-log plot of MSD vs time, with the slopes corresponding to ballistic (dashed line) and diffusive (solid line) trajectories marked. For all larvae, except those of *S. californicum*, the vertical MSD remains ballistic even for long times, whereas the horizontal MSD becomes diffusive.

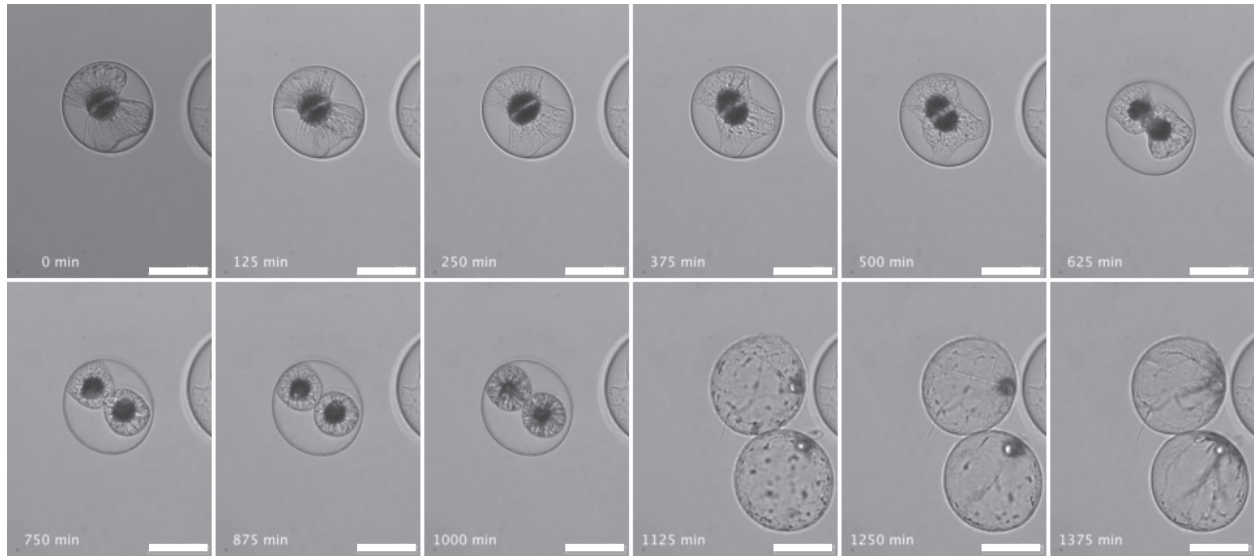


Figure S14 Control experiment with *P. noctiluca*. Snapshots in time of *P. noctiluca* undergoing cell-division observed under a Nikon Ti2-E inverted microscope using DIC imaging. The rapid expansion in the daughter cells volume post-division is seen.

PIV Parameter	Parameter value/range
Pixel size	1.6 μm
Window size	64 pixels
Search area	64 pixels
Overlap	32 pixels
Frame rate	> 30 Hz
Bead size	2 or 3 μm
Bead concentration	0.05% by volume

Table S3 Parameters used for Particle Image Velocimetry (PIV).

411 **3 Supplementary Movie Captions**

Movie 1 Setup and procedure for a vertical tracking experiment.

Movie 2 Multi-scale measurements of sedimenting particles: (1) Interactions in Sedimenting Spheres , (2)Rods, and (3)Microscale transport processes in coupled sedimentation-dissolution.

Movie 3 Tracking of sedimenting marine detritus and concurrent measurement of microscale transport.

Movie 4 A comparative study of marine invertebrate larvae behavior.

Movie 5 Multiscale tracking of freely swimming *P. miniata* (bat-star) larvae reveal behavioral transitions that regulate depth and enable feeding.

Movie 6 Diel behavior of Polychaete larvae measured at the scale of individual organisms.

Movie 7 Measuring of behavior of plankton in depth-patterned virtual environments: Volvox colony response to depth-dependent changes in light intensity.

Movie 8 Tracking single cells: Dynamic sinking behavior in marine diatoms.

Movie 9 Tracking single cells: Measuring cell division and associated changes in sinking rates in the dinoflagellate *Pyrocystis noctiluca*.

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