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Gotta catch ‘em all: Agar microchambers for high-throughput single-cell live imaging

Constraining motile microorganisms for live imaging often requires costly microfluidics or optical traps to keep them in view. We used patterned stamps and agar to make versatile, inexpensive “microchambers” and offer a way to predict the right chamber size for a given organism.

Contributors (A-Z)

Prachee Avasthi, Feridun Mert Celebi, Tara Essock-Burns, Jase Gehring, Megan L. Hochstrasser, Galo Garcia III, David Q. Matus, David G. Mets, Ryan York

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Purpose

As part of an ongoing effort at Arcadia to enable high-throughput image-based comparisons, we need adaptable ways to capture and record the behavior of motile single-celled organisms across a diverse range of species. In a recent pub, we designed microchambers to observe motile cells, including algae and ciliates **[1]**. These 3D-printed microchambers used teflon-coated wells paired with a microscope slide and a polymer coverslip to constrain cells within the field of view (FOV).

While our original designs were useful in some contexts, we wanted to generalize our design to allow 1) more flexibility in chamber size and shape to accommodate different-sized organisms and better organism tracking, 2) the ability to capture single cells or organisms within chambers, 3) construction of the microchambers with fewer parts (easier to make chambers robustly!), and 4) to avoid the need for a 3D printer so that researchers can make chambers without costly machinery.

We turned to inexpensive (~$200–300) custom-fabricated PDMS stamps to mold agar along with standard microscope slides and coverslips.

We demonstrate several different use cases for agar microchambers, including 1) organismal tracking, 2) high-speed sub-cellular imaging, and 3) long-term imaging of unicellular organisms and nematode development. Finally, we provide a testable framework for choosing an ideal microchamber design based on organismal size.

We hope that this resource will be useful for anyone interested in increasing throughput for phenotyping microorganisms with microscopy.

- This pub is part of the **platform effort**, "Microscopy: Visually interrogating the natural world." Visit the platform narrative for more background and context.
- All associated **code** is available in this GitHub repository.
- An accompanying **protocol** on how to make your own agar microchambers is available at protocols.io.
- This pub is a follow-up to our first microchamber resource **[1]**.

The strategy

For unicellular organisms, cell movement is a window into many biological processes, from cytoskeletal organization underlying motility to cell behavior. Our ability to track cell movement in real time is often a rate-limiting step in recording quantitative phenotypic information at single cell resolution. These data are also often noisy at the population-level. While others have used microfluidics **[2]**, micropipetting **[3][4][5]**, or

optical trapping **[6]** approaches to track individual cells, these can be challenging or costly to implement.

We wanted an inexpensive, flexible, and scalable strategy to trap microorganisms for high-throughput measurements. Microwell arrays have previously been used to trap cells for imaging **[7][8][9]** and we adopted this approach to image single organisms behaving in space. After several iterations, we settled on using PDMS micropillar arrays to stamp agarose gel microwells. Here, and in the accompanying protocol, we present a resource for generating and using agar microchambers for live imaging of microorganisms.

The problem: Cells swim out of focus and overlapping tracks make single-cell analysis difficult

This is the second iteration of a microchamber design at Arcadia. The previous design **[1]** works well to capture time-lapse recordings of single-celled organisms at low magnification (e.g. using a 10× objective) but the depth of the water column (~100–600 μm) allows for organisms to leave the FOV during imaging sessions. We noticed this was an issue when analyzing time-lapse recordings of *Chlamydomonas reinhardtii* imaged in teflon microchambers, as we could only track individual algal cells for short timescales (Figure 1, A and A′). While we could theoretically reduce the microchambers to 100 μm in z, the smallest x/y dimensions available were 1 mm × 1 mm. This is quite large relative to the size of the ~10 μm algal cells, making it challenging to isolate single motile cells, requiring cell tracking of many individual cells at once (Figure 1, B and B′). It became clear that we needed a more versatile option.

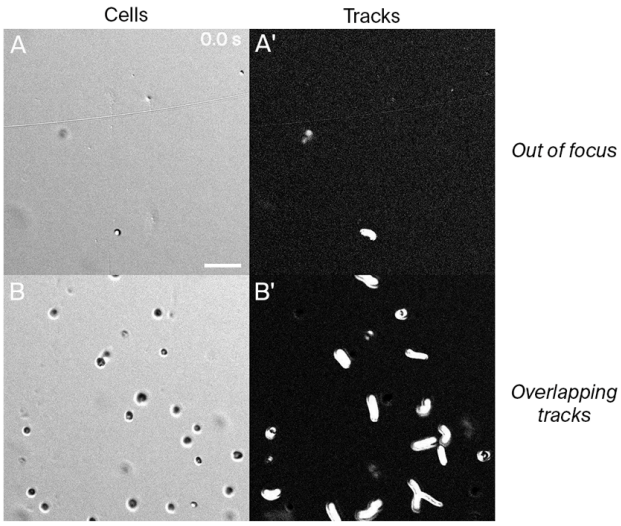

Figure 1

Swimming cells out of focus and overlapping tracks inhibit single-cell studies.

(A) *Chlamydomonas reinhardtii* swimming. The cell near the top of the frame is out of focus — the one near the bottom is in focus. 10× DIC image.

(A′) Cumulative standard deviation projections from beginning of video A. The out-of-focus cell produces a dim track, while the in-focus cell produces a bright track.

(B) *Chlamydomonas reinhardtii* cells swimming with overlapping paths.

(B′) Cumulative standard deviation projections from beginning of video B. Swimming cells produce overlapping tracks. Scale bar = 50 µm. Video is real time.

Our solution: Custom stamps to make flexibly sized microchambers in agar and confine single cells

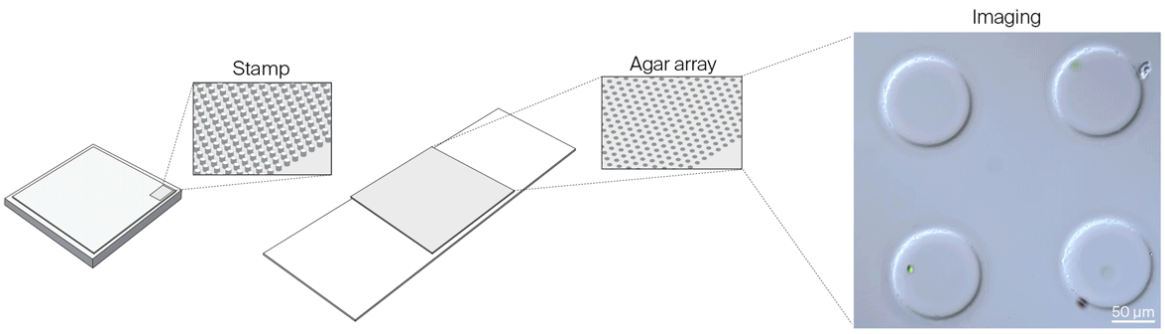

Figure 2

Schematic of stamp and resulting arrayed microchambers in agar for microorganism imaging.

Pairing a PDMS stamp with agar allows us to easily make customizable microchambers to isolate and image motile microorganisms, like the algal cells shown here.

We wanted to generate microchambers that would constrain single swimming microorganisms in x, y, and z, but still allow for freedom of movement within the field of view (FOV). To accomplish this, we generated agar microchambers using pre-made PDMS stamps that we purchased from researchmicrostamps.com (Figure 2). These stamps are inexpensive and available in a variety of shapes and sizes, letting us easily customize microchambers based on cell size and behavior. It is straightforward to capture single cells in individual chambers and simultaneously image many chambers over the course of at least several hours.

The resource

Making microchambers

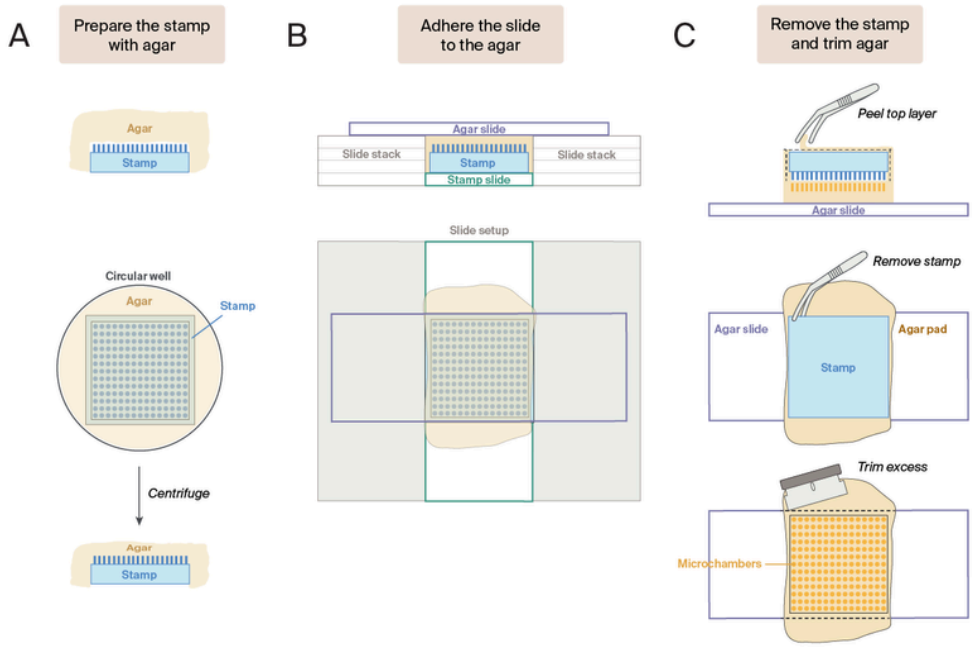

Figure 3

Schematic diagram summarizing key parts of the protocol for making agar microchambers.

(A) Pour liquified agar onto a micropatterned stamp.

(B) Press a slide onto the agar for hardening.

(C) After hardening, carefully remove the stamp and excess agar.

To confine motile algal cells in x, y, and z, we selected a PDMS stamp that would generate 100 µm diameter pools that would be 40 µm deep. After troubleshooting several different methods to generate agar microchambers, we settled on a protocol based on the standard method for generating an agar pad for imaging nematodes (e.g. *Caenorhabditis elegans*), where researchers make an agar pad by sandwiching molten agar between two microscope slides. This strategy of molding agar with a stamp has been useful in a variety of biological applications, such as growth and imaging of

mammalian organoid and spheroid models **[9][10][11]**, imaging motile parasitic *Trypanosoma brucei* **[12]**, and creating arrays for microinjection and subsequent imaging of a variety of animal embryos **[8]**.

In our microchamber protocol, we first centrifuge molten agar with a PDMS stamp (Figure 3, A). This step is often necessary to fully penetrate and wet the PDMS micropillar array with molten agar. Then the stamp with molten agar is placed on a single slide sitting in between a stack of slides to provide depth to the future microchamber (Figure 3, B). After placing another slide perpendicularly on top and allowing the stamp+agar to gel at 4 °C (~30 minutes), we trim the agar and remove the stamp (Figure 3, C). The microchamber is then ready for sample loading by pipetting a small (~1–5 µl) volume of microorganisms in media onto the chamber and subsequent imaging. After several uses, we found that we could skip the centrifugation step, and directly poured molten agar onto the stamp (Figure 3, B). Although there is an increased frequency of merged wells in the microchambers produced in this manner, we were always able to find many intact wells to perform our imaging experiments. We hypothesize that the initial hydrophobic nature of PDMS may require centrifugation for generating microchambers, but over continued use, this seems to be unnecessary. We've provided a detailed version of the protocol (with photos of key steps) on protocols.io. The protocol includes three versions to use depending on whether it is your first time doing the protocol, subsequent times, or the first time using a new stamp.

> **TRY IT**: The full protocol, "Molding microchambers in agar with PDMS stamps for live imaging," is available on protocols.io.

Imaging single organisms in microchambers

Given the difficulties of long-term tracking and an inability to isolate single motile algal cells in our teflon microchambers (Figure 1), we attempted to confine algal cells in microchambers using a PDMS stamp with 100 µm diameter pools that were 40 µm deep. Since we're interested in comparing motility both within and between species at Arcadia, we isolated motile algal cells from two closely related *Chlamydomonas* species, *C. reinhardtii* (Figure 4, A) and *C. smithii* (Figure 4, B). We transferred motile algal cells to fresh agar microchambers made in TAP media and imaged in red-shifted

light [IR long pass, 610 nm (ThorLabs)] to avoid phototactic behaviors for 1–5 minutes using a Kinetix (Photometrics) scMos at 20–50 fps paired with a 10× objective (Nikon, NA 0.45).

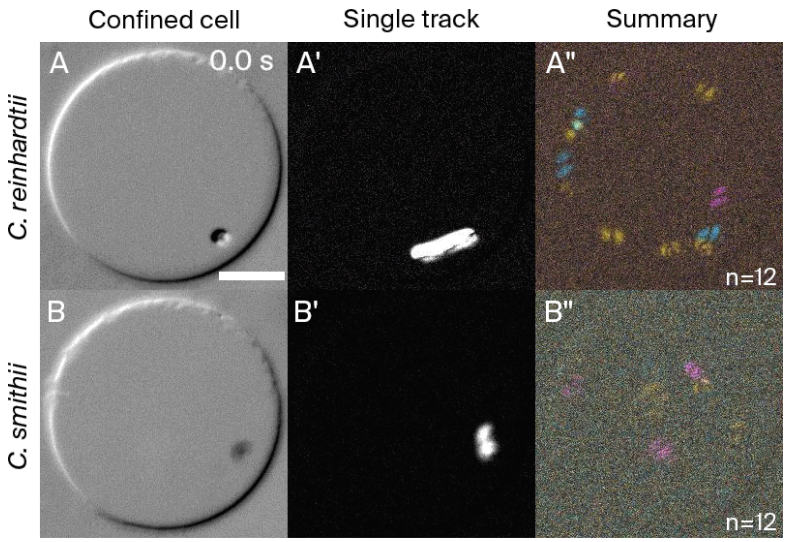

Figure 4

Algal cell confinement in individual microchambers enables comparative studies of motility.

(A) *Chlamydomonas reinhardtii* cell swimming in a 100 µm diameter, 40 µm deep, agar microchamber. 10× DIC image.

(A′) Cumulative standard deviation projections from beginning of video A.

(A″) Summation of tracks from n = 12 motile cells showing that *C. reinhardtii* swims along the edges of the chamber.

(B) *Chlamydomonas smithii* swimming in a microchamber. 10× DIC image.

(B′) Cumulative standard deviation projections from beginning of video B.

(B″) Summation of tracks from n = 12 motile cells showing that *C. smithii* swims in loops, pauses, and

continues swimming in the center of the chamber. Scale bar = 25 µm. Video is 5× fast.

An overlay of multiple recordings reveals that the two algal species display strikingly dissimilar motility patterns in the 100 µm diameter pools, as *C. reinhardtii* spent the majority of time swimming in a circular pattern around the periphery of the microchamber (Figure 4, A″) while *C. smithii* explored much more of the microchamber space (Figure 4, B″). Our results are consistent with recent data from Kirsty Wan's laboratory, examining motility in algal cells captured in microfluidic droplets, where they demonstrate through live imaging and computational modeling that confinement size affects swimming speed and microchamber exploration **[2]**.

Having successfully tracked motile cells in 3D over minute-length time scales, we next asked whether we could use custom agar microchambers to capture finer-scale biology at higher resolution. We hypothesized that in the 100 µm pools, algal cells had freedom to move, well-suited to tracking motility, but that this level of confinement might not be enough to capture sub-cellular structures, such as the flagellar beating that underlies algal motility **[3][4][5]**. To capture flagellar beating in free-swimming algal cells, we made agar microchambers using a 120 × 30 × 30 µm (length × width × height) PDMS stamp (Figure 5). We collected short (~3–5 seconds) DIC time-lapse data at high speed using a Kinetix scMos camera (Photometrics) at 200 fps. Following image processing (see "Materials and methods"), we can visualize flagellar beat patterns in both algal species, shown below in the microchamber (Figure 5, A and B), following image registration (Figure 5, A′ and B′) and as a filmstrip of single frames (Figure 5, A″ and B″) extracted from the movies shown in panels A and B. We're excited to examine these data further to determine whether there are species-level differences in flagellar beat pattern.

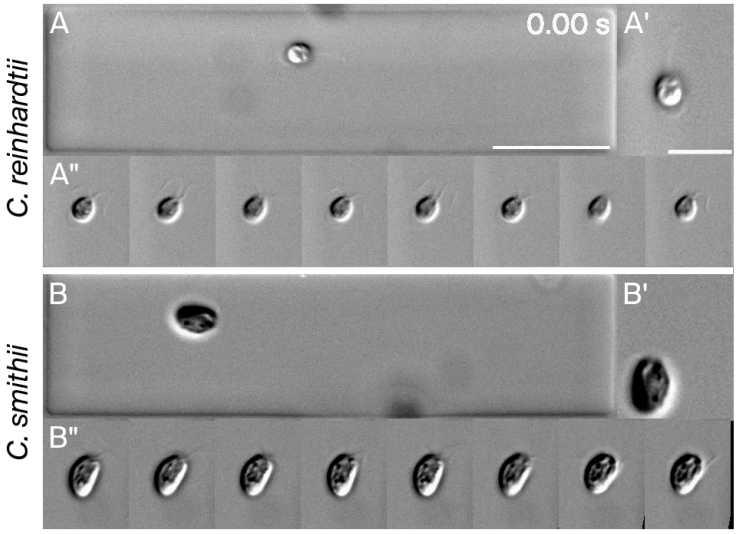

Figure 5

(A and A′) *Chlamydomonas reinhardtii* cell swimming in a 120 µm × 30 µm × 30 µm chamber. 40× DIC image. Video is 12.5× slow.

(B and B') *Chlamydomonas smithii* cell swimming. Similar chamber and imaging conditions as in A.

(A″ and B″) Sequential frames showing *C. reinhardtii* (A″) and *C. smithii* (B″).

Scale bars = 25 µm. Note that in A′ and B', frames are aligned relative to the cell to visualize flagellar beating.

Our agar microchambers work well for both cell confinement and increasing throughput for imaging microorganisms on short timescales (seconds to minutes). However, many biological processes occur over much longer timescales. To test if this simple sample mounting technique also allows for long-term imaging (hours), we performed imaging experiments using 100 × 100 × 40 µm agar microchamber pools with two different organisms. First, we generated long time-lapse recordings of the ciliate, *Colpoda steinii,* as we had previously shown that custom microchambers are useful for visualizing *C. steinii* motility but also to provide a substrate to study adaptation to their environment, such as encystment and excystment **[1]**. Here, we recorded a single isolated, encysted *C. steinii* cell with DIC optics at high magnification

(100×, 1.35 NA) and imaged continuously for three seconds every three minutes for ~two hours (Figure 6). We were able to capture subcellular dynamics (Figure 6, inset) as well as the transition from cyst to a motile state following reanimation.

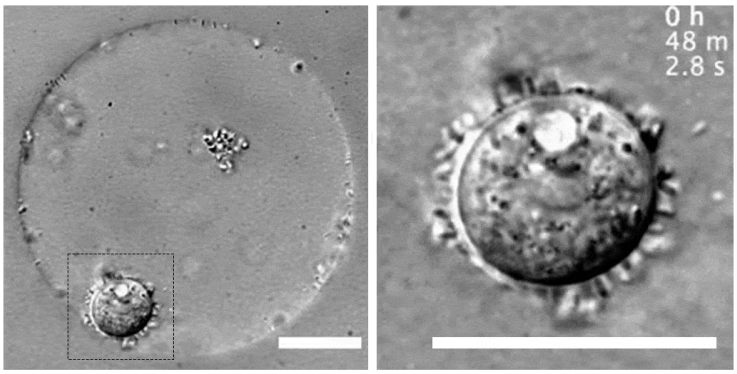

Figure 6

Confinement of *Colpoda steinii* in agar microchambers allows observation of cell behaviors such as reanimation over relevant time windows.

C. steinii cell reanimating from a dormant cyst. Inset shown on right. Five-second movies played 5× fast at three-minute intervals. Scale bar = 25 µm.

Next, we asked whether we could capture the dynamics of embryogenesis. This biology is particularly well-suited to long-term, high-throughput imaging. We performed pilot experiments using the roundworm nematode, *Caenorhabditis elegans*, as their highly stereotyped development is extremely well-characterized **[13]**. We isolated nematode embryos by hypochlorite treatment of gravid adults **[14]** and aliquoted embryos into microchamber arrays. We imaged embryonic development at low (Figure 7, A) and high magnification (Figure 7, B), collecting a single frame every three minutes for ~12 hours using DIC optics. Most isolated nematode embryos developed normally and hatched into L1-stage larvae that were still motile after image collection 12 hours later, suggesting that the microchambers are well-suited for long-term imaging studies.

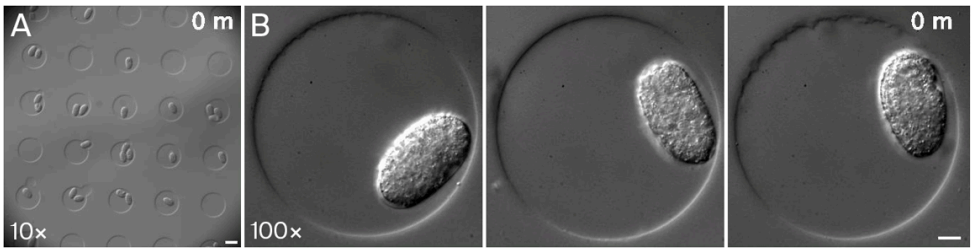

Figure 7

Isolating *C. elegans* embryos in microchamber wells enables high-throughput, long-term imaging of embryonic development.

We isolated mixed-stage embryos and arrayed them into microchamber wells. We collected images every three minutes for ~12 hours using DIC optics at 10× (A) and 100× (B). Scale bar = 50 µm (A) and 10 µm (B).

Impacts of confinement on behavior

One caveat of confining motile organisms is that microchamber dimensions may constrain behavior in a manner that is not physiologically relevant. To examine this, we recorded organism behavior in different-sized microchambers. For ease, we prepared samples from organisms that we had access to in our laboratory, either from established cultures or from environmental samples (see "Materials and methods"). We generated agar microchambers using three different commercially available PDMS stamps (30 × 30 × 30 µm, 100 × 40 µm, and 500 × 500 µm), trapped a range of microorganisms, and collected time-lapse recordings using DIC optics (Figure 8). We isolated several unidentified organisms from a culture of the paramecium, *Paramecium multimicronucleatum* (Carolina Biological Supply), and a water sample from garden flowers. Using our smallest microchamber stamp (30 × 30 × 30 µm), we visualized bacterial motility and cell division (Figure 8, A). We trapped what is likely a *Colpoda* species in a 30 × 30 × 30 µm chamber (Figure 8, B) and an unknown protist in 100 × 40 µm pools (Figure 8, C), to explore how cell behavior differs based on confinement. Using our PDMS stamp with the largest features (500 × 500 µm) we imaged several different unidentified protists (Figure 8, D), an unknown rotifer species (Figure 8, E), and paramecium swimming behavior (Figure 8, F).

Altering the size and shape of individual wells has obvious effects on organismal motility and also allows for different scales of imaging. For example, the *Colpoda* species isolated in the 30 × 30 × 30 μm chamber is unable to swim, but this allows for high-resolution imaging of ciliary beating (Figure 8, B). The paramecium isolated in 500 × 500 μm pools has room to swim, but the z-depth allows it to leave the field of view (Figure 8, F).

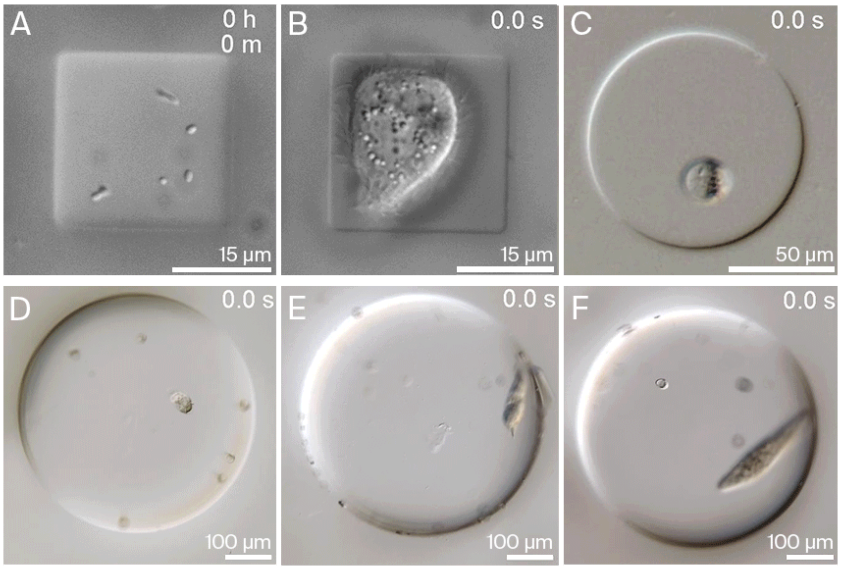

Figure 8

Diverse organisms in varying levels of confinement allow for direct observation of movement behavior.

Notice differences in spatial scale and temporal speed between panels. DIC imaging shows:

(A) Time-lapse video of unknown bacterial species undergoing cell division.

(B) Unknown protist species.

(C) Unknown colpodean species.

(D) Unknown protist species.

(E) *Rotifer*, unknown species.

(F) *Paramecium multimicronucleatum*.

Choosing the right microchamber size for your organism

We wanted a rule of thumb for predicting the ideal microchamber size for an organism of interest. Organismal size is a clear constraint on chamber size. Perhaps less obvious is the effect of an organism's speed of movement. For example, a large organism that swims slowly would likely require a different chamber size from one that swims quickly (since the faster organism would hit the chamber boundaries more frequently).

We decided to come up with a simple formula that would take both species size and behavior into account. We reasoned that we could calculate the area covered by a given organism per second (species speed) and multiply this by an empirically derived factor (essentially a "swimming radius" multiplier) to estimate ideal chamber size.

We can calculate area covered per second (A) as follows, where L is body length (in μm) and S is swimming speed (in μm/s):

$$A = (L/S) \times L$$

In our initial microchamber tests, we found that the ideal chamber size for *C. reinhardtii* (i.e. the smallest size at which we still observed cell swimming consistent with open-field behavior) is 100 μM in diameter (24,674 μm in area). If we divide this area by *C. reinhardtii*'s area covered per second, we get 189.8, which we rounded neatly to 200. We use this as our "swimming radius" multiplier to calculate the maximum area (Y):

$$Y = A \times 200$$

From this, we can extract the minimum chamber diameter (M, in μm):

$$M = (\sqrt{Y}/\pi) \times 2$$

We performed these calculations on a publicly available resource, the BOSO-Micro dataset **[15]**, which contains information about cell size, shape, and behavior for 382 unicellular species/cell types of swimming prokaryotes and eukaryotes, and plotted the results (Figure 9). Referring to this plot should provide a decent starting point in choosing the right microchamber size for a new organism, but will likely involve a bit of trial and error as well.

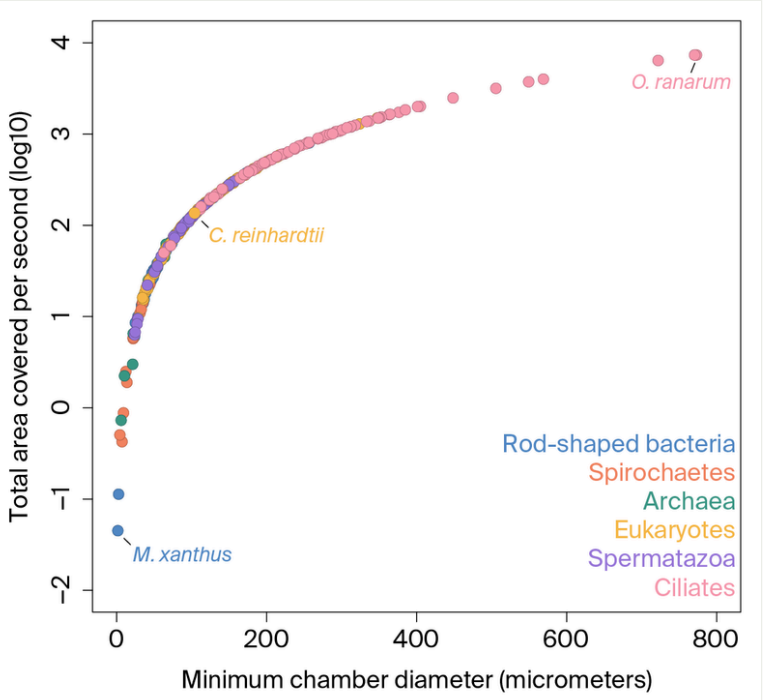

Figure 9

We can estimate minimum chamber size as a function of swimming cell size and behavior.

Estimated minimum chamber size is plotted in comparison to the total area covered/second (log10 scale). Total area covered combines cell size and speed information to get a sense of how much physical space a cell can explore in a given second. Here, we hypothesize that an ideal chamber size for open-field swimming experiments should be >200× the area covered in a second. Points are colored by taxonomic class. Exemplary species are highlighted, including *Chlamydomonas reinhardtii*.

Materials and methods

Read our detailed methods below or skip to the "Key takeaways" section.

Preparing agar microchambers

In addition to the brief description at the beginning of "The resource," we've written up a detailed, step-by-step protocol that explains how to make microchambers and includes photos of key steps.

> **TRY IT**: The full protocol, "Molding microchambers in agar with PDMS stamps for live imaging," is available on protocols.io.

Organism maintenance

C. reinhardtii and *C. smithii*

We obtained wild-type *C. reinhardtii* (cc124) and *C. smithii* (cc1373) from the Chlamydomonas Resource Center (University of Minnesota). We maintained clonal populations from the stock streaks by live transfer once per week in 3 mL of tris-acetate-phosphate (TAP) medium. We grew liquid cultures at room temperature with light and agitation.

C. steinii

We cultured *Colpoda steinii* (American Type Culture Collection 30920) by weekly reanimation of resting cysts with filtered (0.2 µm) hay medium supplemented with an overnight inoculum of *Klebsiella aerogenes* (American Type Culture Collection 13048). On the day after reanimation, we inoculated culture supernatant containing swimming *Colpoda* into a vessel containing fresh hay medium and bacteria. We incubated cultures in a humid chamber at 25 °C **[1]**.

C. elegans

We maintained wild-type *C. elegans* strain N2 on NGM plates on an OP50 *E. coli* lawn using standard methods **[16]**. We isolated embryos by hypochlorite treatment of gravid adults **[17]** and pipetted onto agar microchambers for imaging.

Other microorganisms

We isolated unknown Colpodean and bacterial species from water in a flower vase containing stem-cut lilies obtained at Berkeley Bowl West in Berkeley, CA. We obtained *Paramecium multimicronucleatum*, rotifer, and unknown protist species from Carolina Biological Supply.

Microscopy

We performed imaging on either a Nikon Ti2-E & Yokogawa CSU W1-SoRa microscope, equipped with an ORCA-Fusion BT digital scMOS camera or a Nikon Ni-E microscope equipped with a Photometrics Kinetix digital scMos camera. We performed differential interference contrast (DIC) imaging using a Plan Apo 10× 0.45 Air objective, a Plan Apo Lambda S 40× NA 1.25 Silicone immersion objective, or an SR HP Plan Apo Lambda 100× NA 1.35 Silicone immersion objective, as indicated.

Image processing

We acquired images using the Nikon NIS Elements software and files were in an ND2 format. We imported these files into FIJI (ImageJ) **[18]** as TIFF files, where we split and merged channels, and selected a subset of frames. We registered images of algae (Figure 5) using the FIJI plugin StackReg **[19]**. Other image processing methods are described in more detail in our associated GitHub repository. We used ChatGPT to add comments to our code.

Microchamber size estimation

We used a publicly available data set **[15]** to calculate the minimum microchamber diameter for a range of species based on their body length and swimming speed, as described in detail above. The associated code is available on GitHub.

All **code** associated with this pub is available in this GitHub repository (DOI: 10.5281/zenodo.7893571).

Key takeaways

It is our hope that this resource and the accompanying protocol will provide a means to explore cell behavior in microorganisms. By combining commercially available PDMS stamps, agar, and common laboratory equipment, researchers can make custom microchambers to isolate microorganisms to study single cell behavior, community interactions, or tissue level processes such as embryonic development and morphogenesis.

By bypassing the need for microfluidics or complex optical trapping equipment, these custom agar microchambers should lower the barrier to exploring microorganism behavior temporally as well as imaging distributions of behavior within populations and across the tree of life.

Next steps

Based on the results shown here, there are several areas of research that we're excited to explore. Now that we can isolate single organisms in individual microchambers, we plan to collect high-dimensional data on a diverse range of phenotypes. We are interested in making comparisons across the tree of life and would like to extend our work into a multicellular framework, which was a motivating factor in testing whether we could isolate nematode embryos for live imaging to unlock additional phenotypic and evolutionary comparative space.

We also need to gain a better understanding of how confinement affects cell behavior, to both be able to make equivalent comparisons between datasets and to match organismal size, motility, and cell behavior with microchamber shape and size. One obvious area of improvement that would better facilitate these next steps would be to increase our ability to customize microchamber stamp designs. We are interested in exploring this through custom 3D printing and would welcome feedback or suggestions on this. While the array of commercially available stamps at

researchmicrostamps.com allows for a wide range of chamber types, we can imagine wanting further customization, which could become costly and difficult to iterate quickly in the lab.

Finally, we are excited to see how our protocol for generating microchambers will be useful to the scientific community and would welcome feedback on this resource and the accompanying protocol. If you use this resource and the accompanying protocol, we would love to hear about your specific use cases as well as any relevant feedback you have or ideas for further improvements.

Acknowledgements

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