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## Motility Patterns of *Stentor* During and After Oral Apparatus Regeneration

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### Abstract

*Stentor coeruleus* has long been employed as a model organism to understand unicellular regeneration. Transcriptomic analysis of individual cells revealed hundreds of genes -- many not associated with the oral apparatus -- differentially regulated in phases throughout the regeneration process. We hypothesized that this systemic reorganization and mobilization of cellular resources towards growth of a new oral apparatus will lead to observable changes in movement and behavior corresponding in time to the phases of differential gene expression, thus allowing an assay of functional regeneration based on motility analysis. However, the morphological complexity of *Stentor coeruleus* meant the development of an assay to capture the statistics and timescale was necessary. Using a custom script to track cells in short videos, we compile statistics over a large population ( $N \sim 100$ ) to show that, upon loss of the OA, *Stentor coeruleus* initially loses the ability for directed motion, then starting at approximately four hours exhibits a significant drop in speed until approximately eight hours. This assay provides a useful tool for the screening of motility phenotypes, and can be adapted for the investigation of other organisms.

### SUMMARY:

We present a useful assay and data visualization method for characterization of motility and behavior of a population of cells. Here, this assay reveals that *S. coeruleus* transitions through four behaviorally distinct phases when regenerating a lost oral apparatus.

### Keywords

*Stentor coeruleus*; motility; regeneration; phenotype screening; microscopy; timelapse

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A complete version of this article that includes the video component is available at <http://dx.doi.org/10.3791/62352>.

### DISCLOSURES:

The authors have nothing to disclose.

## INTRODUCTION:

The mechanisms by which cells attain complex shapes and structures is still poorly understood. The giant ciliate *Stentor coeruleus*, (*Stentor*) has long been employed as a model organism to understand unicellular morphogenesis and regeneration owing to its large size, conspicuous surface patterning, resilience to a vast array of microsurgical techniques, and ease of culturing in a laboratory setting<sup>1-3</sup>. Early regeneration studies focused on the oral apparatus (OA), the largest and most morphologically distinct feature in *Stentor*, which is shed in its entirety upon chemical shock<sup>4-6</sup>. *De novo* replacement of a lost OA begins with emergence of a new membranellar band which gradually moves into position at the anterior of the cell before reforming into a functional OA over eight morphological stages<sup>3</sup>. These stages have been observed sequentially regardless of temperature and provide a universal reference point for nearly all studies since<sup>6</sup>. Mechanistic analysis of *Stentor* regeneration requires tools for measuring the timing of regeneration that is robust and simple enough to be applied to multiple samples as part of a chemical or molecular screen.

The standard method for implement a cell-based assay would be to use imaging, in this case to image formation of new OA during regeneration. However, such imaging-based assays are most effective when the regenerating structure contains distinct molecular components that can be used as markers, such that they would be easily detected in a fluorescence image. In the case of the *Stentor* OA, the known components (cilia, basal bodies) are also present on the rest of the cell surface, such that recognizing the restoration of the OA can not be done simply by looking for the presence or absence of a component. Rather, some form of shape recognition would be required to detect an OA, and this is potentially very challenging given the fact that *Stentor* cells often change shape via a rapid contractile process. Here we present an alternative assay for regeneration which relies on the motile activity of the body and OA cilia. As the OA regenerates, the newly formed cilia undergo reproducible changes in position and activity, which in turn affects the swimming motility of the cell. By analyzing motility, it is possible to implement an assay for “functional regeneration”, that quantifies regeneration by quantifying the function of the regenerated structures.

Previous analysis of *Stentor* ciliary function during regeneration used particle image velocimetry (PIV) to observe changes in flow pattern at different stages of regeneration (Wan 2020), but this approach requires laborious imaging of individual cells one at a time. By using the motion of the cell itself as a proxy for cilia-generated flow, it would be possible to analyze larger numbers of cells in parallel, using low-resolution imaging systems compatible with high throughput screening platforms. This assay can, in principle, be used to study development and functional regeneration in other swimming organisms.

Section 1 of the protocol describes the construction of a multi-well sample slide which allows for high through-put imaging of a population of cells over up to an entire day. Details are provided for how to adjust for use with other cell types. Section 2 of the protocol covers the acquisition of video data for this assay, which can be accomplished on a dissection microscope with a DSLR camera. Section 3 of the protocol provides a walk-through of cell tracking and cell speed calculation using included MATLAB code. Section 4 of the protocol

explains how to turn the numerical results into plots as shown in Figure 1C–F and Figure 2B for easy interpretation of results.

## PROTOCOL:

A population of approximately one hundred *S. coeruleus* cells were cultured in accordance with previously published JoVE protocol<sup>7</sup>.

### 1. Sample Preparation

- 1.1. Cut a piece of 250  $\mu\text{m}$  thick silicone spacer sheet (Table of Materials) slightly smaller in both height and width than a microscope slide.
- 1.2. Using a 5/16" hole punch, create circular wells. Be mindful of leaving sufficient space between neighboring wells to ensure a good seal -- we found 3 mm to be sufficient. With practice, ten wells can be placed on a single sample slide.
- 1.3. Initiate regeneration of oral apparatus by incubating the cells in 10% sucrose or 2% urea for two minutes. Then wash three times with fresh media<sup>7</sup>.
- 1.4. Gently pipette approximately ten stentor into each well. Be careful of not overfilling the well volume.

### 2. Visible light microscopy time lapse

- 2.1. Place the sample onto the microscope stage and set magnification to the lowest available such that one well fits in frame in its entirety.
- 2.2. Begin timelapse. Acquire a ten second video of each well at each timepoint. If using a microscope setup with a motorized X-Y stage, the entire timelapse can be automated. Otherwise, a user needs to be present at each timepoint to manually translate the sample.
- 2.3. Note: Avoid leaving light on to avoid heating and evaporation. The sample volumes are small and evaporation will lead to air bubbles.
- 2.4. Acquire a clear image of a calibration slide or a clear ruler for pixel to millimeter conversion.
- 2.5. Save movies as TIFF, MOV, or AVI. (These are the most common non-proprietary video file types. Depending on the specific microscope software, the videos may save by default to a proprietary file type, but then can be exported to one of the aforementioned file types.)

### 3. Cell tracking

- 3.1. Download the two MATLAB scripts TrackCells.m and CleanTraces.m to an easy-to-remember location on a computer with MATLAB installed. If the data videos are not already on this computer, transfer them onto this computer. The data videos and the scripts do not need to be in the same folder.
- 3.2. Organize data videos into folders, one for each timepoint.

- 3.3. Use the script TrackCells.m first to perform automated cell tracking in the data videos. Open TrackCells.m in the MATLAB editor window by double-clicking the file.
- 3.4. Click the green arrow at the top of the MATLAB window to run the script.
- 3.5. When prompted, “Select One or Data Videos to Initiate Tracking”, navigate to the data videos you had acquired during Step 2. You may select multiple video files by using shift-click, control-click, or by holding down the left mouse button while moving over the files to highlight them.
- 3.6. Once satisfied with the list of files in the “File name:” box at the bottom of the prompt window, click “Open”.
- 3.7. This script will now create a folder for each video file chosen. It will then write into this folder each frame of the video as a .jpg file and a file named position\_estimates.mat which contains all traces found in the video.
- 3.8. Depending on the size of the videos, the number of videos, and the speed of the computer, this script can take hours to run. It is recommended that a test run be done on one video first to confirm all parameters are set correctly (See Discussion below).

#### 4. Trace verification

- 4.1. Verify that Step 3 was done correctly by checking that MATLAB did not give any error messages before proceeding.
- 4.2. Use the CleanTraces.m script to manually reject anomalous or false traces. Open CleanTraces.m in the MATLAB editor window by double-clicking the file.
- 4.3. At the prompt “Select data folder output by TrackCells.m. It will have the same name as the video file.”, navigate to one of the folders created in Step 3. Choose only one folder.
- 4.4. This script will now display the traces one-by-one in a pop-up window. They are overlaid on the frame of the video where the trace starts, in green, and the frame of the video where the trace ends, in magenta. Therefore, a valid trace should link a green cell and a magenta cell.
- 4.5. In the main MATLAB screen, enter 1 to keep the trace and 0 to reject the trace. Press enter to move on to the next trace.
- 4.6. New traces will continue to display until either there are no more traces, or the first sixty have been shown. When this process is complete, the script will automatically create a folder named “CLEAN TRACES” for saving the outputs and display all remaining traces on top of the first frame of the video. This image is automatically saved as “LabeledTraces.png” for future reference.
- 4.7. MATLAB will also save all traces the user had chosen to keep in the file “clean\_traces.mat”.

- 4.8. Complete this step for all videos in one time point before continuing. For the data in this manuscript, one video was acquired for each well at each timepoint, for a total of ten videos per timepoint.

## 5. Data visualization

- 5.1. To visually compare the motility of the entire cell population across different timepoints, we translate all traces from Step 4 to begin at the origin and create one plot for each timepoint (Figure 1C–F, see S1 for all time points). To do this, begin by downloading the MATLAB script titled “SpaghettiPlots.m”.
- 5.2. Double-click “SpaghettiPlots.m” to open the script in MATLAB editor. Click the green arrow in the top toolbar to run.
- 5.3. A window file browser window will pop-up with the prompt “Select time folder containing well data (clean\_traces.mat) to graph.” Navigate to the folder of one of the time points. Note that this folder should contain within it a folder for each of the analyzed videos.
- 5.4. When prompted “Calibration: What is the number of pixels per millimeter?”, use the calibration image with any picture viewer to count the number of pixels corresponding to one millimeter physical distance, and enter into the MATLAB command window. For this step, the authors use FIJI/ImageJ with plot profile command. Press enter.
- 5.5. The script will now combine the traces from all of the analyzed videos at this time point into a single plot. Faint dotted circles in the plot indicates radial displacements of 1, 2, 3, and 4mm, respectively.
- 5.6. Adjust axes of the plot as necessary by changing line 55 of the script, which by default is set to “rr = 4” for including up to the radius equals 4 mm circle.
- 5.7. Save the plot.

## REPRESENTATIVE RESULTS:

In this control assay, a gradual and sustained increase in movement range from the most motile cells is observed within the regenerating *Stentor* population. At two hours, the earliest time point, no cells move more than 1mm during the ten second video, while at nine hours many cells traversed more than 5mm during the same length of time. We note that there is one caveat to the interpretation of this example series of plots. The fastest swimming cells were able to swim across the well within the video, especially if by chance they started close to the edge; thus they are restricted from swimming entirely in a straight line and their movement range, as visualized in this way, will appear smaller. While this does complicate any attempts to quantify the distance traveled by the cells, it does not alter the qualitative trend that the range of movement increases through at least eight hours. This is consistent with the known morphological regeneration timeline<sup>3</sup>.

Further inspection of the tracked motion revealed a gradual transition from directionless to directed swimming patterns across the population (Figure 1G). Directionless swimming

traces appear as circles or arcs, and were only observed prior to Y hours. Stentors are asymmetrical along the anterior-posterior axis and the dorsal-ventral axis. This asymmetry necessitates a spiral swimming motion which in our centroid tracking code displays as a sinusoidal trajectory [14]. While healthy *Stentor* possess cilia not just along the membranellar band but also all over the surface of the body along cortical rows, only the oral cilia have been associated with motion control. It has been observed that inversion of the beat direction of these cilia correlate with inversion of swimming direction [3]. Cells in this study were individually inspected under a microscope immediately after sucrose shock to confirm that they had fully shed their membranellar band. Therefore, movement during 2–3 hours post sucrose-shock results entirely from body cilia, which explains its adirectional, haphazard nature. The gradual increases in both range and directionality suggest that the membranellar band contributes to cellular motility hours before complete regeneration.

It is known that primordial cilia first reappear approximately three hours into oral apparatus regeneration, but not until around 6.5–8.5 hours does the beating synchronize across the OA, forming metachronal waves<sup>8</sup>. In healthy stentor, the OA generates long range flows -- extending multiple body-lengths -- in a characteristic dual vortex pattern which serves to corral edible particles towards the OA for ingestion into food vacuoles. In this context, our assay shows that when the regenerating membranellar band is short, swimming direction is skewed towards one side of the cell. Therefore, early on in regeneration, *Stentor* swim in a small circular pattern, sometimes using their holdfast as the pivot point. As the membranellar band elongates, forward momentum increases however combined with their asymmetric shape, their swimming will continue to skew in one direction forming progressively larger and larger circular patterns. With a fully formed and positioned membranellar band, asymmetric spinning still occurs; however, coordinated, large, beating cilia from the MB results in overall forward movement with a slight rotation along the body axis.

To compile population statistics we classified the tracked cells into three distinct categories, which we refer to as non-motile with no visible holdfast, non-motile with visible holdfast, and motile. These categories of behavior were previously defined by Tartar, but to our knowledge never quantified for their prevalence over time<sup>3</sup>. We do so with our dataset in Figure 2A. At all time points we found over half of the cell population were not observably motile. Additionally, at later times, a significant number of the cells were found in colonies with visible holdfasts, strongly suggesting they had explored the environment and preferentially attached near others of its kind. An example of this can be seen in the final inset of Figure 2B.

Statistics on average speed for individual cells is shown in Figure 2B. We observed four behaviorally distinct phases in this population. These phases are described below, with transcriptional context from the work of Sood *et. al.* and Onsbring *et. al.*<sup>9, 11</sup>

### Phase 1 (0– 3 hours):

Cells display little to no visible motion and visually lacks morphology. The range of movement of the most motile cells in the population gradually increase during this phase, while the cell speeds were surprisingly varied, as seen in the wide spread in the scatter

overlay of Figure 2B. Immediately subsequent sucrose shock, the cell undergoes wound closure<sup>9</sup> and only the body cilia remain. During this time, the cell is known to be repressing transcription factors suggesting that the cell has recognized that it is missing the oral apparatus. Aurora kinases, which are required for microtubule polymerization<sup>10</sup>, are among the genes that are repressed<sup>11</sup>. This suggests that the cell is deconstructing its cytoskeleton in order to begin rebuilding. With this knowledge, we can begin hypothesizing that this deconstruction leads to the decrease in movement.

#### **Phase 2 (4–7 hours):**

While range of movement continued to increase through this phase, as seen in Figure 1, cell speeds drop across the population as seen in Figure 2B. It is known that during this portion of OA regeneration, cyclins, ciliary motility and chromatin regulators are differentially expressed. The genes that are differentially expressed are centriole biogenesis, cell cycle regulators. At this point, centriole biogenesis genes are actively transcribed and this is confirmed both visibly and implicitly through lack of swimming.

#### **Phase 3 (8–10 hours):**

A statistically significant increase in speed was observed between hours 7 and 8. The fastest cells often swam across the well or even followed the wall all the way around during a ten second video. We interpret this to be an intentional exploration of the environment, as many of these cells ceased the movement after settling into a small colony of cells or finding a gap between an air bubble and the edge of the well. From transcriptomics, it is known that kinases, kinesins, and zinc finger proteins are differentially expressed during this time.

#### **Phase 4 (>10 hours):**

The cell population transitioned from exploring the environment to securely anchored with their holdfast, and extended in Stentor's characteristic trumpet shape. As the sample wells contain only pacified spring water besides the cells, there is no food present. Nonetheless, the cells at this phase behaviorally resemble healthy cells in a nutrient-rich environment. No transcriptomic study of stentor regeneration to date had been carried out beyond 9 hours, so little is known about the genomic activity. However, our results would suggest that the cells have fully regenerated by this time and few genes should be differentially regulated during this phase.

## **DISCUSSION:**

### **Critical steps in the protocol**

It is critical for this assay, especially when used to investigate motility over many hours, that the silicone spacer in the imaging chamber has a secure seal to both the glass slide and the coverslip. Failure to do this will result in wells leaking into each other or air bubbles forming inside the wells. For researchers unfamiliar with the use of silicone spacers, we strongly recommend that PSW or other clean medium be pipetted into each well to test for leakage before use.



### Modifications and troubleshooting of the method

The dimensions of the sample wells were all chosen to work well for *S. coeruleus*, which are ~ 1 mm in size. This includes silicone spacer thickness (chosen to constrain the *S. coeruleus* cells under investigation to a two-dimensional plane), 5/16" diameter, and ten cells per well. These numbers should be adjusted when adapting this protocol for other cell types.

If the MATLAB code returns traces which do not match well with the cell movement in the data video, check that the intensity threshold for increasing image contrast is set correctly. When incorrectly set, the cell outlines will be poorly identified or missed altogether, thereby preventing the tracking to be done correctly. If no intensity threshold works well for correct tracking, try with a video with higher contrast or is more in focus.

### Limitations of the method

The data analysis of this assay relies on tracking cells from the acquired videos with high fidelity. As such, our code has difficulty following cells which are temporarily visually obstructed by another cell, and when multiple cells spend multiple frames in close proximity -- situations which are difficult for any particle tracking program. Fortunately the frequency of the former can be effectively reduced simply by limiting the number of cells placed inside one well. As for the latter, *S. coeruleus*'s preference to settle in colonies caused some of these cells not to be identified by our code. However, as all of these cells exhibit little to no motion, valid statistical comparisons could still be drawn between motile cells across all our experimental datasets.

### The significance of the method with respect to existing/alternative methods

The current tool kit for *Stentor* includes surgical dissection, osmotic shock, RNAi<sup>4</sup>, DNA/RNA analysis and small molecule inhibitors. Having a pipeline that provides the movement rate of the cells would have added to previous publications and will be used in future publications to analyze phenotypes. For example, Mob1 knockdown resulted in the formation of malformed and multiple mouths with inappropriate cilia placement. Had this technique to read out the ability to swim been available, there would be more evidence towards the argument that Mob1 is used in repatterning the cilia, used for directional swimming, and cortical rows in *Stentor*. For future phenotypes, this high throughput way of capturing swimming will add another degree of characterization thus broadening the way we study *Stentor*.

### Importance and potential applications of the method in specific research areas

Future questions - One fascinating question that can be addressed is cellular decision making, how does a cell decide to literally, "sink or swim?" How does *Stentor* decide on a place to anchor and attach their holdfast? From Tartar and our observations, given enough agitation, *Stentor* will decide to detach from their holdfast and begin to swim. This binary decision-making ability in a single-cell shows that a neuron is not necessary to choose. Probing this question requires a method to measure movement, which is provided by this manuscript.

## Supplementary Material

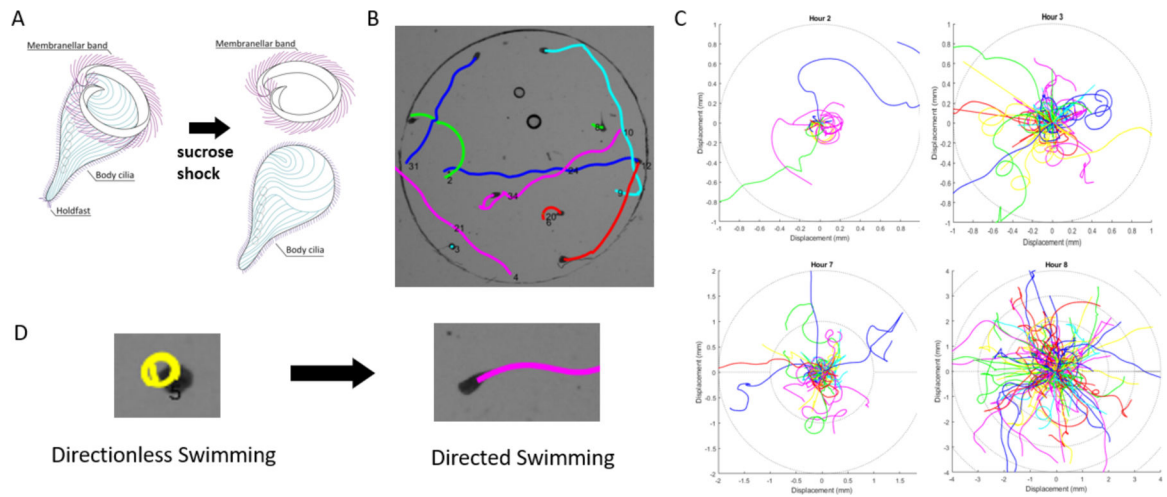
Refer to Web version on PubMed Central for supplementary material.

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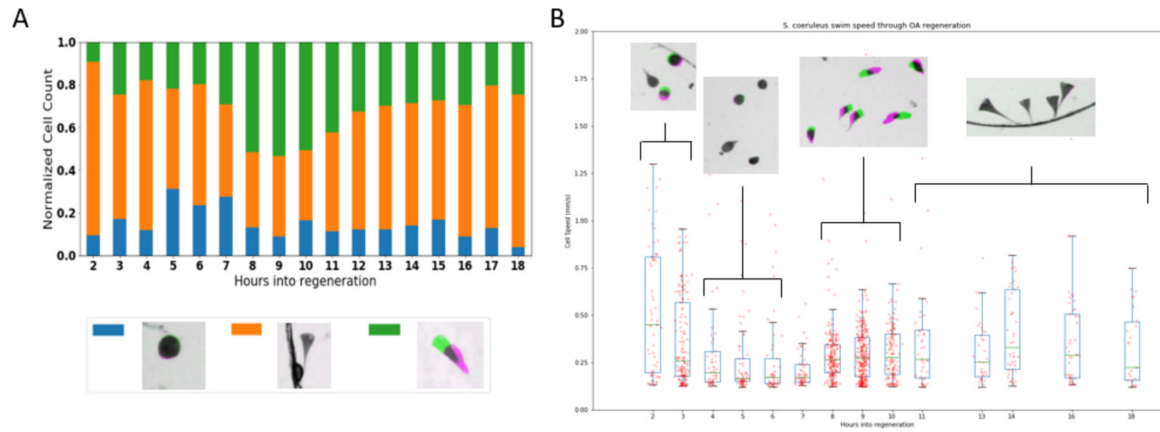
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**Figure 1: Cell tracking reveals gradual recovery of directed swimming.**

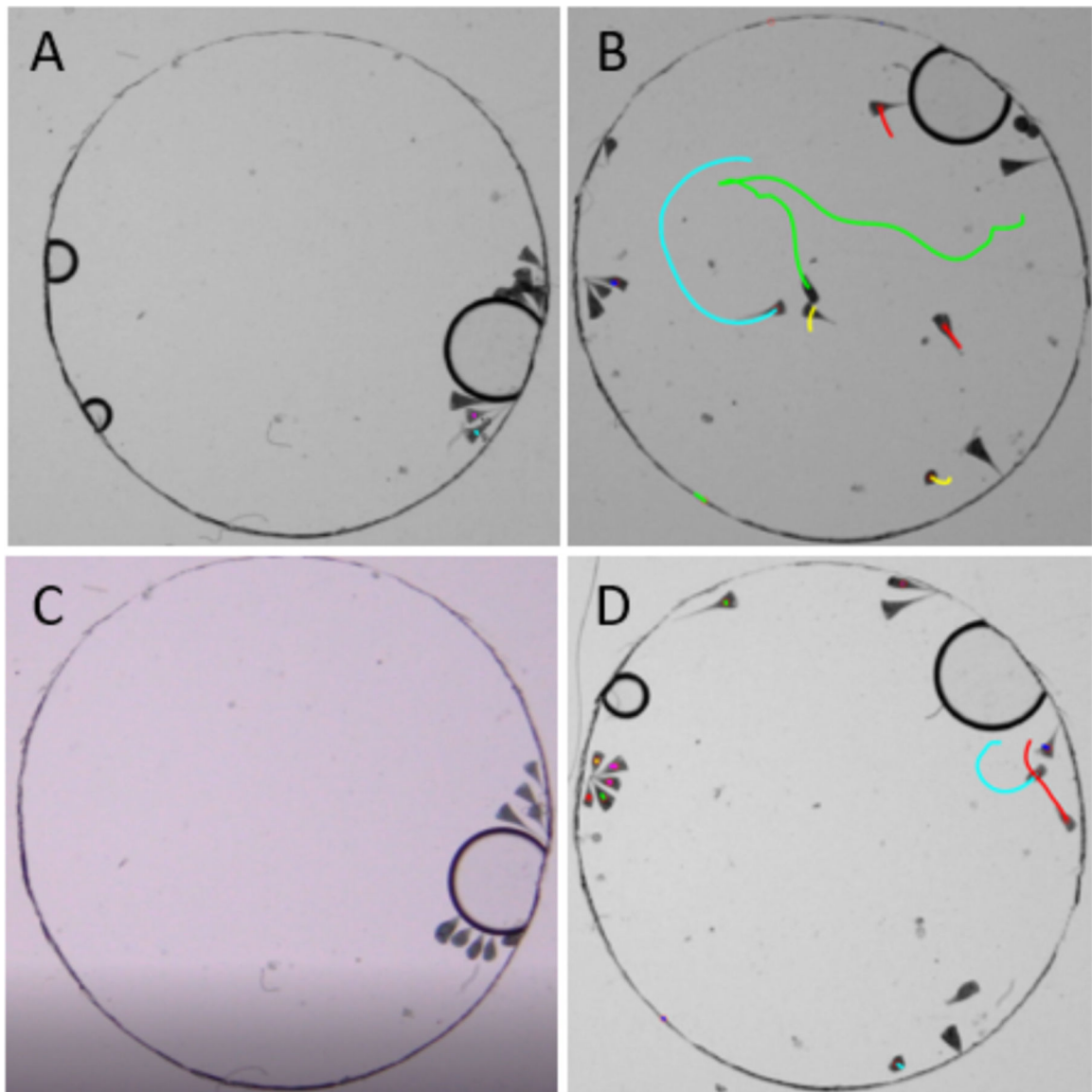
**A.** Sucrose shock induces *S. coeruleus* to shed membranellar band. **B.** Example tracking result of regenerating cells in a ten second duration video. **C-F.** Overlay of all trajectories at 2 hours, 3 hours, 7 hours, and 8 hours. Times were rounded to the closest hour during compilation of data. Dotted circles indicate radial displacements of one millimeter. **G.** Cell motility evolves from directionless swimming characterized by short circular trajectories, to directed swimming characterized by long sinusoidal trajectories.



**Figure 2: Motile subpopulation demonstrates four distinct phases of behavior through OA regeneration.**

**A.** Normalized counts of cells at each time which were (blue) not motile, with no visible holdfast; (orange) not motile, with visible holdfast; (green) motile. Legend shows representative cells under each category as visualized by MATLAB function `imshowpair`.

**B.** Boxplot of swim speed; boxes extend from Q1 to Q3 quartile values at each time with median value indicated in green. Scatter overlay are the average speed of each motile cell. Inset shows representative population behavior during each of four phases.



**Figure 3: Motility analysis as a phenotype screening tool requires large numbers of cells.**

**A, B.** Tracking results from two wells at twelve hours. **B-D.** Tracking results from the same two wells at eighteen hours. Stentors display a preference to attach in clusters, with some wells settling into colonies hours sooner than others.

## Materials

Name	Company	Catalog Number	Comments
0.25 mm-thick silicone sheet	Grace Bio-Labs	CWS-S-0.25	
24 x 50 mm, #1.5 coverglass	Fisher Scientific	NC1034527	As noted in Discussion, smaller coverglass can be used if fewer sample wells are placed on one slide.
CCD camera			We used Nikon D750
Chlamydomonas 137c WT strain	Chlamydomonas Resource Center	CC-125	
MATLAB	MATHWORKS		
MATLAB Image Processing Toolbox	MATHWORKS		needed for TrackCells.m and CleanTraces.m

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