Journal of Visualized Experiments

Analysis of Motility Patterns of Stentor During and After Oral Apparatus Regeneration Using Cell Tracking

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE62352R1
Full Title:	Analysis of Motility Patterns of Stentor During and After Oral Apparatus Regeneration Using Cell Tracking
Corresponding Author:	Janet Sheung Claremont Colleges UNITED STATES
Corresponding Author's Institution:	Claremont Colleges
Corresponding Author E-Mail:	JSheung@kecksci.claremont.edu
Order of Authors:	Janet Sheung
	Megan Otsuka
	Athena Lin
	Gabriella Seifert
	Eliana Smithstein
	Wallace Marshall
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please specify the section of the submitted manuscript.	Biology
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Claremont, CA, USA
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please provide any comments to the journal here.	
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)

TITLE:
Analysis of Motility Patterns of *Stentor* During and After Oral Apparatus Regeneration Using Cell Tracking

AUTHORS AND AFFILIATIONS:
Janet Y. Sheung[1], Megan Otsuka[2], Gabriella Seifert[3], Athena Lin[4], Wallace F. Marshall[4]

[1]W. M. Keck Science Department, Scripps, Pitzer, and Claremont McKenna of The Claremont Colleges, Claremont, California 91711, USA
[2]W. M. Keck Science Department, Pitzer College, Claremont, California 91711, USA
[3]W. M. Keck Science Department, Scripps College, Claremont, California 91711, USA
[4]Department of Biochemistry and Biophysics, School of Medicine, University of California at San Francisco, San Francisco, California 94158, USA

Email addresses of co-authors:
Megan Otsuka (motsuka@students.pitzer.edu)
Gabriella Seifert (gseifert5779@scrippscollege.edu)
Athena Lin (athena4@stanford.edu)
Wallace F. Marshall (Wallace.Marshall@ucsf.edu)

Corresponding author:
Janet Y. Sheung (jsheung@kecksci.claremont.edu)

KEYWORDS:
Stentor coeruleus, motility, regeneration, phenotype screening, microscopy, time-lapse, cilia

SUMMARY:
We present a protocol for the characterization of motility and behavior of a population of hundred micron- to millimeter-sized cells using brightfield microscopy and cell tracking. This assay reveals that *Stentor coeruleus* transitions through four behaviorally distinct phases when regenerating a lost oral apparatus.

ABSTRACT:
Stentor coeruleus is a well-known model organism for the study of unicellular regeneration. Transcriptomic analysis of individual cells revealed hundreds of genes—many not associated with the oral apparatus (OA)—that are differentially regulated in phases throughout the regeneration process. It was hypothesized that this systemic reorganization and mobilization of cellular resources towards growth of a new OA will lead to observable changes in movement and behavior corresponding in time to the phases of differential gene expression. However, the morphological complexity of *S. coeruleus* necessitated the development of an assay to capture the statistics and timescale. A custom script was used to track cells in short videos, and statistics were compiled over a large population (N ~100). Upon loss of the OA, *S. coeruleus* initially loses the ability for directed motion; then starting at ~4 h, it exhibits a significant drop in speed until

~8 h. This assay provides a useful tool for the screening of motility phenotypes and can be adapted for the investigation of other organisms.

INTRODUCTION:

Stentor coeruleus (*Stentor*) is a well-known model organism that has been used to study unicellular regeneration owing to its large size, ability to withstand several microsurgical techniques, and ease of culturing in a laboratory setting[1–3]. Early regeneration studies focused on the largest and most morphologically distinct feature in *Stentor*—the OA—which is shed completely upon chemical shock[4–6]. *De novo* replacement of a lost OA begins with the emergence of a new membranellar band—an array of cilia that gradually shift towards the anterior of the cell before forming a functional OA over eight morphological stages[3]. These stages have been observed sequentially, regardless of temperature, and provide a universal reference point for nearly all studies[5].

Mechanistic analysis of *Stentor* regeneration requires tools for measuring the timing of regeneration that are robust and simple enough to be applied to multiple samples as part of a chemical or molecular screen. The standard method for performing a cell-based assay is imaging, in this case, imaging the 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, so 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; therefore, recognizing the restoration of the OA cannot be achieved 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. This paper presents an alternative assay for regeneration that 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 perform 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, combined with tracer beads added to the external media, to observe changes in flow pattern at different stages of regeneration[7]; however, this approach requires laborious imaging of individual cells and their associated flow fields, 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 in the hundreds of microns to millimeters size scale. Section 1 of the protocol describes the construction of a multiwell sample slide, which allows for high-throughput 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 digital single-lens reflex camera. Section 3 of the protocol provides a walk-through of cell tracking and cell speed calculation using MATLAB code (**Supplemental Information**). Section 4 of the protocol explains how to turn the numerical results into plots as shown in **Figure 1C–F** and **Figure 2C** for easy interpretation of results.

PROTOCOL:

NOTE: A population of approximately one hundred *S. coeruleus* cells were cultured in accordance with a previously published JoVE protocol[8].

1. Sample preparation

1.1. Cut a piece of 250 µm-thick silicone spacer sheet (**Table of Materials**) slightly smaller in both height and width than a microscope slide. Using a 5/16" hole punch, create circular wells. Be mindful of leaving sufficient space between neighboring wells to ensure a good seal.

NOTE: A space of 3 mm between neighboring wells was found to be sufficient. With practice, ten wells can be placed on a single sample slide.

1.2. Initiate regeneration of OA by incubating the cells in 10% sucrose or 2% urea for 2 min (**Figure 1A**). Then, wash three times with fresh medium[8]. Gently pipette approximately ten *Stentor* into each well. Be careful of matching sample volume to the well volume as closely as possible.

NOTE: For the well dimensions used here, 12.5 µL of sample was pipetted into each well.

1.3. Close the wells by gently lowering a piece of coverglass (see **Table of Materials**) over the wells starting from one edge. Use a narrow and slightly flexible object, such as a 10 µL pipette tip, to press down on the coverglass where it contacts the silicone sheet, to ensure a good seal.

2. Visible light microscopy time-lapse

2.1. Place the sample on the microscope stage, and set magnification to the lowest available such that one well fits in frame in its entirety.

NOTE: A 1.6x camera adaptor and 1x magnification on the objective were used on the microscope for this protocol.

2.2. Begin time-lapse. Acquire a 10 s video at 30 frames per second of each well at each timepoint. If using a microscope setup with a motorized X-Y stage, automate the entire time-lapse. Otherwise, ensure that a user is present at each timepoint to manually translate the sample to record each well.

NOTE: Avoid leaving the sample illuminated when not imaging to avoid heating and evaporation.

The sample volumes are small, and evaporation will lead to air bubbles.

2.3. Save movies as TIFF, MOV, or AVI.

NOTE: 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.

2.4. Use a pixel to millimeter conversion factor for physical scale, and perform a calibration or use known pixel size from the camera used. To calibrate, acquire a clear image of a calibration slide or a clear ruler at the same magnification settings as the videos. Using any image viewing program, count the number of pixels between marks of a known physical distance.

NOTE: For example, if the image of a ruler shows the 1 mm mark and 2 mm mark to be separated by 100 pixels, the conversion factor is 1 mm per 100 pixels. Alternatively, to derive this factor from camera pixel size, simply multiply the camera pixel size by the magnification. For example, if the camera used has 3.45 µm pixels, and the magnification used was 1.6x, then the conversion is 3.45 µm * 1.6 = 5.52 µm per 1 pixel.

3. Cell tracking

3.1. Download the two scripts, **TrackCells.m** and **CleanTraces.m**, to an easy-to-remember location on the computer. If the data videos are not already on this computer, transfer them onto this computer.

NOTE: 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. Use the script **TrackCells.m** first to perform automated cell tracking in the data videos. Open **TrackCells.m** and run the script.

3.3. Choose **Add to Path** if prompted by a pop-up window., which will typically only happen when the script is run for the first time from a given folder. When prompted, **Select One or More Data Videos to Initiate Tracking**, navigate to the data videos (section 2). 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.

3.4. Once satisfied with the list of files in the **File name:** box at the bottom of the prompt window, click on **Open**. Perform a test run on one video first to confirm all parameters are set correctly (see discussion below).

NOTE: 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. Depending on the size of the videos, the number of videos, and the speed of the computer, this script can take hours to run.

4. Trace verification

4.1. Verify that steps described in section 3 were followed correctly by checking that there are no error messages before proceeding. Use the **CleanTraces.m** script to manually reject anomalous or false traces. Open **CleanTraces.m** in the **MATLAB editor** window by double-clicking on the file.

4.2. 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 as described in section 3. Choose only one folder.

NOTE: 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.

4.3. When prompted, enter **1** to keep the trace and **0** to reject the trace. Press **Enter** to move on to the next trace.

NOTE: 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 (**Figure 1B**). This image is automatically saved as **LabeledTraces.png** for future reference. All traces the user had chosen to keep will be saved in the file **clean_traces.mat**.

4.4. Complete this step for all videos in one time point before continuing.

NOTE: 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

NOTE: To visually compare the motility of the entire cell population across different timepoints, all traces from section 4 were translated to begin at the origin and create one radial displacement versus time plot for each timepoint (**Figure 1C–F**, see **Supplemental Figure S1** for all time points).

5.1. Begin by downloading the script titled **SpaghettiPlots.m**. Open and run the script. When a window file browser window pops 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.

5.2. When prompted **Calibration: What is the number of pixels per millimeter?**, type in the calibration value found in step 2.4, and press **Enter**.

NOTE: The script will now combine the traces from all the analyzed videos at this time point into a single plot (**Figure 1C–F**). Faint dotted circles in the plot indicate radial displacements of 1, 2, 3, and 4 mm.

5.3. Adjust axes of the plot as necessary by changing line 55 of the script, which by default, is set to **rr = 4** for including a radius of up to 4 mm. Save the plot.

REPRESENTATIVE RESULTS:

The goal of this assay is to quantify the gradual change of movement patterns and phased increase in movement speed from cells within a large (N ~100) regenerating *Stentor* population. To aid interpretation of results, the custom code included in this protocol generates two types of plots: an overlay of all cell movement traces in a set of video data (**Figure 1C–F** and **Figure S1**), and a plot of swim speed by hour since the start of regeneration (**Figure 2C**). A population of *Stentor*, which are regenerating normally, should demonstrate a steady transition from directionless swimming to directed swimming (**Figure 1G**). While each cell undergoes this transition in full during the OA regeneration process spanning ~8 h, variation between individuals necessitates looking at the movement of a large population in aggregate for trends to become clear.

Figure 1C–F show the collective motility of the same population of *Stentor* cells at 2, 3, 7, and 8 h into the OA regeneration process. These plots show both the expected increase in number of motile individuals (number of visible traces) and the four-fold increase in range of the most motile cells within the population (radial displacement). It is important to note that axes are different between the different panels of **Figure 1C–F**, with 1 mm (Hour 2), 2 mm (Hours 3 and 7), and 4 mm (Hour 8) chosen as most appropriate to showcase the motion. At 2 h—the earliest time point—no cells move more than 1 mm during the 10 s video, whereas at 9 h, many cells traversed more than 5 mm during the same length of time. **Supplemental Figure S1** provides these plots for the full time-lapse, with identical axes used across all plots to give a clearer view of the expected increase of motility in a successful experiment.

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 over at least 8 h. This is consistent with the known morphological regeneration timeline (**Figure 2A**)[3].

To compile population statistics, the tracked cells were classified into three distinct categories: non-motile with no visible holdfast, non-motile with visible holdfast, and motile. These categories of behavior were previously defined by Tartar, but never quantified for their prevalence over time[3]. **Figure 2B** shows a stacked histogram summarizing relative cell counts across these categories as a function of hours into regeneration. At all time points, over half of the cell population was 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 their kind. An example of this can be seen in the final inset of **Figure 2C**.

$$t_{12} = \frac{x_1 - x_2}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

where x_1 and x_2 denote the mean speed during Phases 1 and 2, respectively; s_1 and s_2 denote the standard deviations of the speed during Phases 1 and 2, respectively; and n_1 and n_2 denote the number of traces extracted during Phases 1 and 2, respectively. The resulting *t*-statistic t_{12} can then be translated into a p-value for hypothesis testing. For the data shown in **Figure 2C**, the transitions from Phase 1 to Phase 2 ($p < 0.1\%$) and from Phase 2 to Phase 3 ($p < 0.1\%$) are statistically significant, whereas the transition from Phase 3 to Phase 4 ($p > 20\%$) is not. It is important to note that the tendency for the cells to settle into small colonies during Phase 4 (**Figure 2B**, inset), as is apparent in the raw videos, is one example of behavioral difference not well captured by measurement of swim speeds alone. This explains the large standard deviation measured during Phase 4 (0.26 mm/s), which in turn, contributed to a lower *t*-statistic when comparing it against Phase 3.

FIGURE AND TABLE LEGENDS:

Figure 1: Cell tracking reveals gradual recovery of directed swimming. (**A**) Sucrose shock induces *Stentor coeruleus* to shed membranellar band. (**B**) Example tracking result of regenerating cells in a 10 s video with striped circles indicating air bubbles in the well. (**C–F**) Overlay of all trajectories at 2 h, 3 h, 7 h, and 8 h. 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**) Morphology of *Stentor* regeneration. (**B**) Normalized counts of cells at each time that were (blue) not motile, with no visible holdfast; (orange) not motile, with visible holdfast; (green) motile. Multiple datasets that span different subsets of hours were combined for this figure, so total cell count per hour ranged from 57 to over 376. Legend shows representative cells under each category as visualized by false color, in magenta and green. (**C**) Boxplot of swim speed; boxes extend from Q1 to Q3 quartile values at each time with median value indicated in green. The scatter overlays are the average speed of each motile cell. Inset shows representative population behavior during each of the four phases. Abbreviations: OA = oral apparatus; Q1 = first quartile; Q3 = third quartile.

Figure 3: Motility analysis as a phenotype screening tool requires large numbers of cells. (**A, B**) Tracking results from two wells at 12 h. (**C, D**) Tracking results from the same two wells at 18 h. *Stentor* cells display a preference to attach in clusters, with some wells settling into colonies hours sooner than others. Striped areas indicate air bubbles in the well.

Table 1: Comparison of swim speeds during each phase of motility recovery.

Supplemental Figure S1: Population swimming patterns through regeneration and beyond. (**A–M**) Motion of *Stentor coeruleus* cells 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 13, 14, 16, and 18 h, respectively, after the initiation of oral apparatus regeneration.

Supplemental Videos 1–3: Example microscopy videos of *S. coeruleus* for script debugging. Section 5 of protocol can be performed on this set of three data videos for a quick confirmation that the scripts are running correctly. In addition, these videos provide a concrete example of contrast and resolution requirements for the data videos.

DISCUSSION:

Many particle and cell tracking algorithms currently exist, some entirely free. Cost and user-friendliness are often trade-offs requiring compromise. Additionally, many of the existing cell-tracking programs are designed to track slow crawling motion of tissue culture cells, rather than the fast swimming motion of *Stentor*, which rotates while swimming and can undergo sudden changes of direction. After testing many of these options, the protocol presented here is intended to be a one-stop solution to go all the way from data acquisition to data visualization using only low-cost equipment and a single scientific software package (**Table of Materials**). This is of particular interest to research labs at teaching-focused institutions or biophysics teaching labs where most of the needed equipment is already available, as it lowers the barrier for students to ask their own questions and arrive at quantitative results within a short timeframe.

The method presented in this manuscript can be adapted to quantitatively characterize the motility of a population of organisms in the hundred microns to millimeter size scale. Application to smaller or larger organisms will require changes to how the video data must be acquired. As applied to a population of regenerating *S. coeruleus* here, this protocol yielded a timeline to the functional recovery of motility across the cell population, which complements what is currently known about the transcriptional timeline. For example, it is known from RNA sequencing that synthesis of genes encoding ciliary motility proteins does not take place until several hours into the regeneration process, long after the formation of the cilia themselves[9, 10]. The statistically significant drop in motility from hours 0–3 (Phase 1) to hour 4 (Phase 2) demonstrated above is consistent with this lag in expression of ciliary motility factors compared to the expression of genes involved in ciliary assembly. No transcriptomic study of *Stentor* regeneration to date had been carried out beyond hour nine, so little is known about the genomic activity. The lack of statistically significant difference between hours 8–10 (Phase 3) and hours 11+ (Phase 4) suggests that the cells have fully regenerated by this time, and few genes relevant to cell motility should be differentially regulated beyond hour 9.

Two major limitations to this method are its failure to track clustered and/or touching cells, and its inability to quantify more complex aspects of motion than swim speeds or trajectories. All particle/cell tracking algorithms have difficulty following cells that are temporarily visually obstructed by another cell, and when multiple cells spend multiple frames in close proximity; the script included here is not an exception. Fortunately, the frequency of the former can be effectively reduced simply by limiting the number of cells placed inside one well as discussed in the next paragraph. Specifically for the *S. coeruleus* population investigated here, their preference to settle in colonies (**Figure 3**) caused some of these cells not to be identified by the script. However, as all these cells exhibit little to no motion, statistics on the motile cell population is unaffected. Additionally, videos where multiple cells elude tracking are easy to identify and manually exclude from further analysis. Regarding the quantification of complex aspects of motion, the transition from directionless to directed swimming seen in **Supplemental Figure S1** suggests quantitative changes in the time correlation of direction, average acceleration, and potentially other measurables. Speed and range, the only quantities analyzed in this protocol, represent only a small aspect of motion.

Lastly, a few parts of the protocol are of particular importance or consequence in practical use. Assembly of the multiwell sample slides with thin silicone sheets, as described in protocol section 1, requires practice. It is easy to introduce leaks or air bubbles into at least one well while sealing the sample with the coverglass. The wells can be divided over multiple sample slides to allow the use of smaller coverglass, which will make the process easier (and mistakes less costly). Though the assay presented here can be used to characterize the motility patterns of a cell population at a single timepoint, it is demonstrated here as a tool to compare motility patterns of a single population over a long time-lapse (over 10 h). As with any long time-lapse, evaporation of a wet sample is unavoidable. Evaporation can be minimized by a secure seal of the silicone spacer in the imaging chamber 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, pasteurized spring water or other clean medium should be pipetted into each well to test for leakage before use. The dimensions of the sample wells were all chosen to work well for *S. coeruleus*, which are approximately 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 10 cells per well. These numbers should be adjusted when adapting this protocol for other cell types.

Several parameters in the **TrackCells.m** script can be tweaked to optimize automatic cell identification and trace validation. The parameters **MinCellArea** and **MaxCellArea** (Lines 15 and 16 of script) allow the user to set the acceptable range of sizes for a cell in square pixels. What values are best depends on many details of the experiment including cell size, camera pixel size, magnification, and whether there are objects which could be misidentified as cells, for example, air bubbles. The default values of 300 and 1500 were optimal for the exact equipment and settings provided in the protocol. After **MinCellArea** and **MaxCellArea** have been optimized, tune the parameter **Threshold** (Line 17) to change image contrast. When incorrectly set, the cell outlines will be poorly identified or missed altogether, thereby hindering correct tracking. If no value of **Threshold** works well for correct tracking, try with a video with higher contrast or that

is more in focus. Line 218 of the script, **bad_trks = find(strk_trks > 20);** is responsible for discarding traces that deviate from the predicted motion (via Kalman filter) too many times. As is, the script has this threshold for **too many** set at **20**. Decrease this integer value to as far as 1 to discard traces more aggressively. Increase the value to discard less aggressively. Much of **TrackCells.m** was adopted from the multi-object tracking tutorial freely accessible at http://studentdavestutorials.weebly.com/multi-bugobject-tracking.html, which the reader could refer to for more detail. **Supplemental Videos 1–3** are example data videos for a test-run of the scripts.

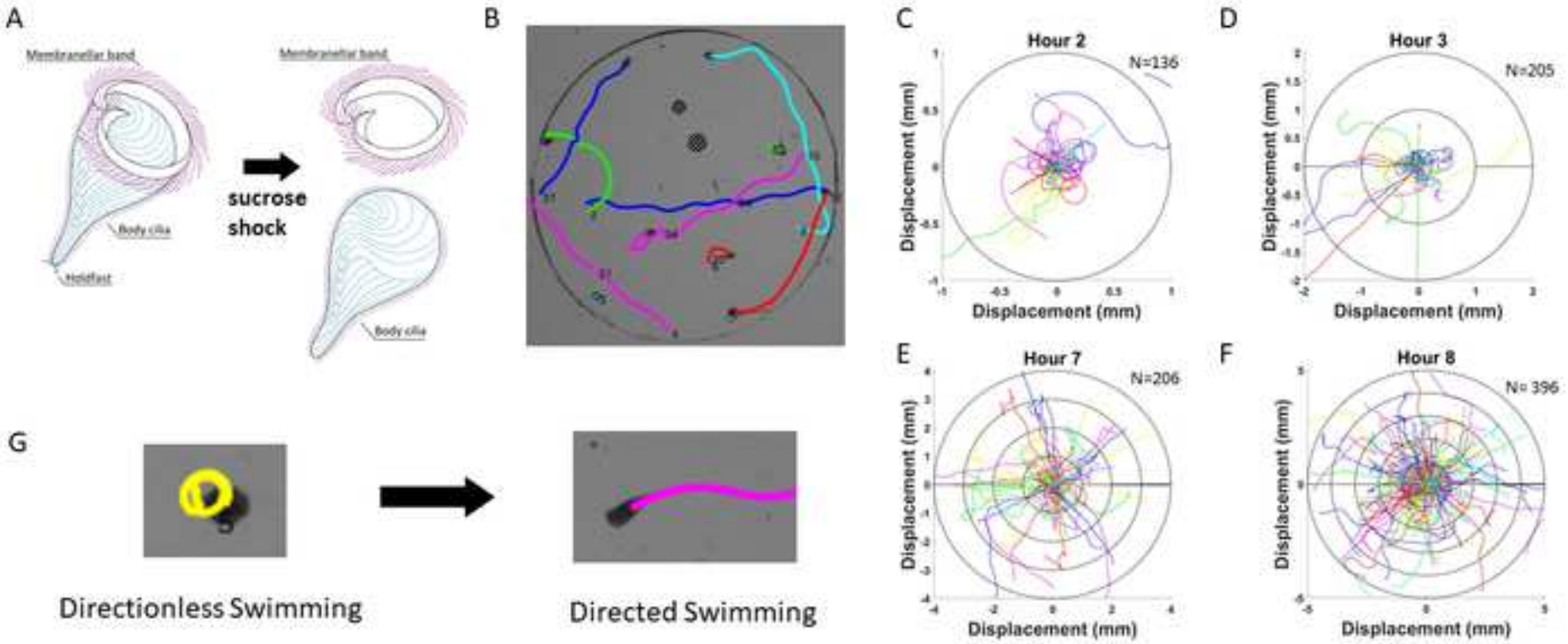
The ability of *S. coeruleus* to fully regenerate a lost OA has been first observed over a century ago, yet many open questions remain regarding the recovery of motility and behavioral states. The protocol presented here is intended to demystify the combination of brightfield imaging, automated cell identification and tracking, and data visualization the authors employed to quantitatively characterize the motility of a population of regenerating *Stentor*. This workflow is broadly applicable to the investigation of motility in general, and the scripts and protocol included can be readily adopted to work with other biological systems of similar size and speed, for example, other ciliates such as *Paramecium* and *Blepharisma*. Furthermore, changing the imaging chamber (*e.g.*, using dry wells or different size wells coupled with different imaging magnification) greatly expands the applicability of this workflow. The current tool kit for *Stentor* includes surgical dissection, osmotic shock, RNAi, DNA/RNA analysis, and small molecule inhibitors[6]. The high-throughput method of quantifying motility presented here adds a complementary degree of characterization and will be used in future work to investigate motility phenotypes.

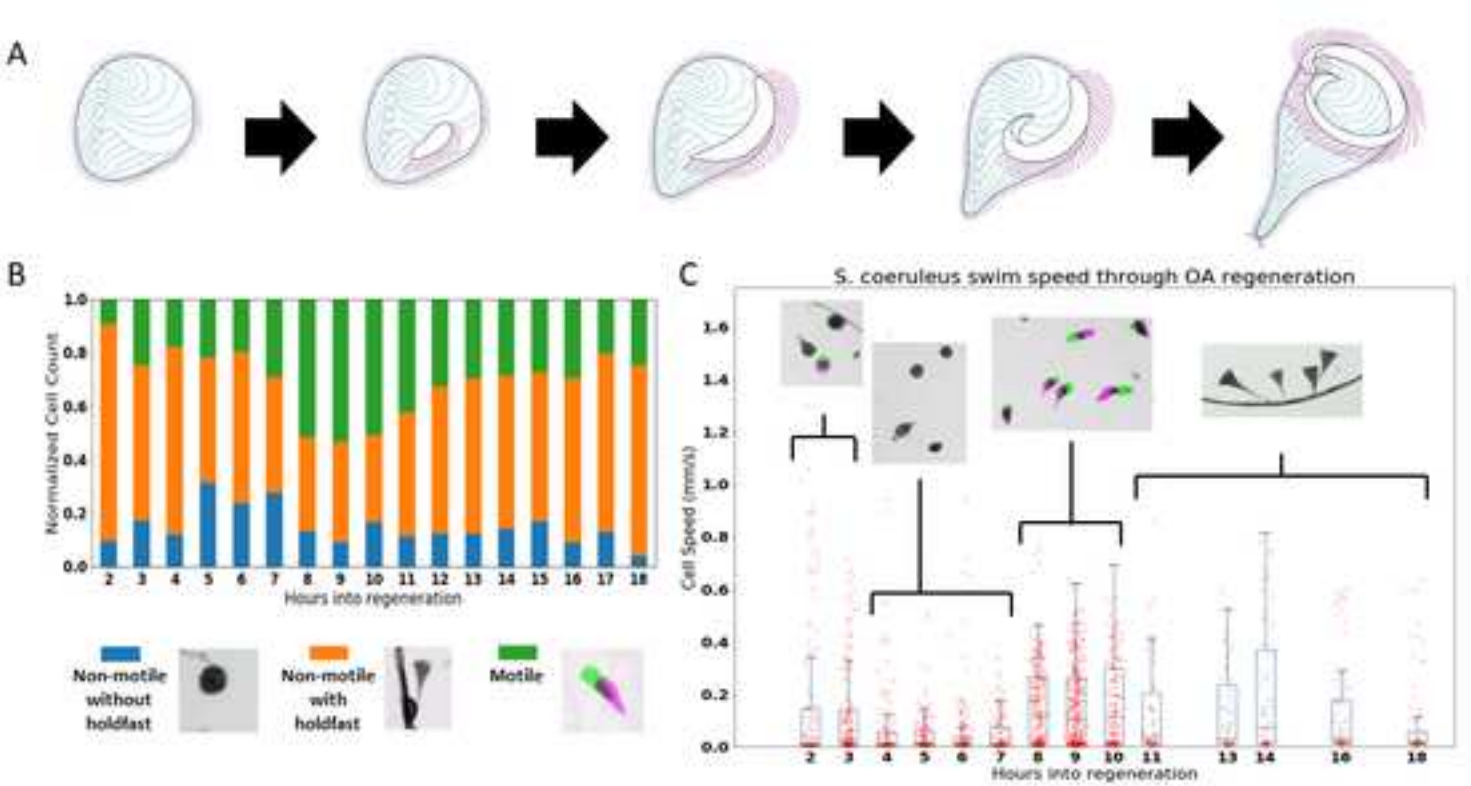
ACKNOWLEDGMENTS:
This work was supported, in part, by Marine Biological Laboratory Whitman Early Career Fellowship (JYS). We acknowledge Evan Burns, Mit Patel, Melanie Melo, and Skylar Widman for helping with some of the preliminary analysis and code testing. We thank Mark Slabodnick for discussion and suggestions. WFM acknowledges support from NIH grant R35 GM130327.

DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:
1. Lillie, F. R. On the smallest parts of stentor capable of regeneration; a contribution on the limits of divisibility of living matter. *Journal of Morphology*. **12** (1), 239–249 (1896).
2. Morgan, T. H. Regeneration of proportionate structures in *Stentor*. *The Biological Bulletin*. **2** (6), 311–328 (1901).
3. Tartar, V. *The Biology of Stentor*. Kerkut, G. A. (Ed), Pergamon Press (1961).
4. Tartar, V. Reactions of *Stentor coeruleus* to certain substances added to the medium. *Experimental Cell Research*. **13** (2), 317–332 (1957).
5. Kelleher, J. K. A kinetic model for microtubule polymerization during oral regeneration in *Stentor coeruleus*. *Biosystems*. **9** (4), 269–279 (1977).
6. Slabodnick, M. M. et al. The kinase regulator Mob1 acts as a patterning protein for *Stentor*

morphogenesis. *PLOS Biology*. **12** (5), e1001861 (2014).
7. Wan, K. Y. *et al.* Reorganization of complex ciliary flows around regenerating *Stentor coeruleus*. *Philosophical Transactions of the Royal Society B: Biological Sciences*. **375** (1792), 20190167 (2020).
8. Lin, A., Makushok, T., Diaz, U., Marshall, W. F. Methods for the study of regeneration in *Stentor*. *Journal of Visualized Experiments : JoVE*. (136), 57759 (2018).
9. Sood, P., McGillivary, R., Marshall, W. F. The transcriptional program of regeneration in the giant single cell, *Stentor coeruleus*. *bioRxiv*. 240788, doi: 10.1101/240788 (2017).
10. Onsbring, H., Jamy, M., Ettema, T. J. G. RNA sequencing of *Stentor* cell fragments reveals transcriptional changes during cellular regeneration. *Current Biology*. **28** (8), 1281–1288.e3 (2018).





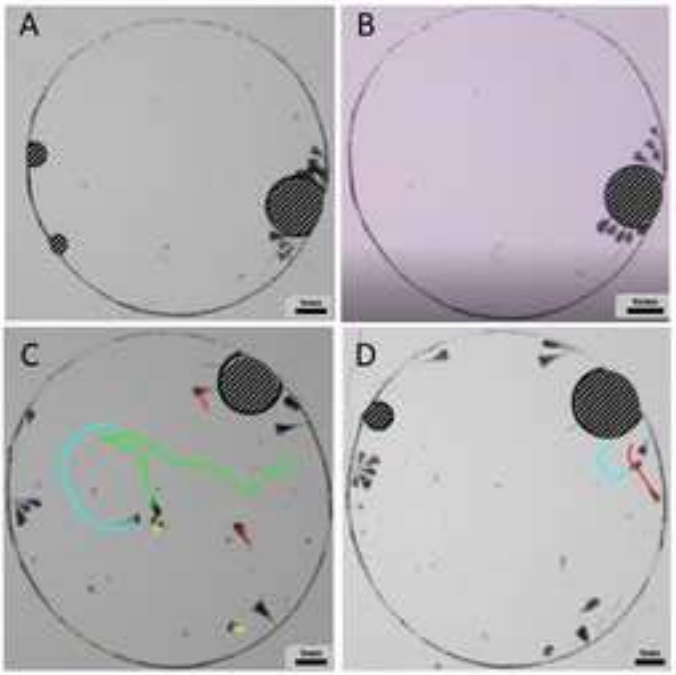


Table 1

	Mean (mm/s)	Standard Deviation (mm/s)
Phase 1	0.14	0.24
Phase 2	0.06	0.13
Phase 3	0.16	0.16
Phase 4	0.15	0.26

Table of Materials

Name of Material/ Equipment	Company	Catalog Number
0.25 mm-thick silicone sheet	Grace Bio-Labs	CWS-S-0.25
24 x 50 mm, #1.5 coverglass	Fisher Scientific	NC1034527
CCD camera		
Chlamydomonas 137c WT strain	Chlamydomonas Resource Center	CC-125
MATLAB	MATHWORKS	
MATLAB Image Processing Toolbox	MATHWORKS	
MATLAB Statistics and Machine Learning Toolbox	MATHWORKS	
Microscope with camera port		
Pasteurized Spring Water	Carolina	132458
TAP Growth Media	ThermoFisher Scientific	A1379801

Comments/Description

As noted in Discussion, smaller coverglass can be used if fewer sample wells are placed on one slide.
We used Nikon D750

needed for TrackCells.m and CleanTraces.m
needed for TrackCells.m
We used Zeiss AxioZoom v1.6 and Leica S9E

Can also be made for much cheaper following recipe from Chlamy Resource Center

February 21, 2021

Re: Resubmission of manuscript *Analysis of Motility Patterns of Stentor During and After Oral Apparatus Regeneration Using Cell Tracking,* JoVE62352

Dear Vidhyer:

We thank you and the referees for the detailed and constructive comments, as well as the opportunity to revise our manuscript. We respond below with the original comments in black and responses in red. We believe these changes have substantially improved the clarity of the manuscript and hope that it is now suitable for publication in the Journal of Visualized Experiments.

This revision has been developed in consultation with all co-authors and each has given approval for this resubmission. Thank you for your consideration.

Regards,

Janet Sheung, PhD

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The manuscript has been read over more than once and multiple corrections made.
2. Please provide an institutional email address for each author.
3. Please provide the complete addresses of the affiliations.

The authors and affiliations section has been edited to provide the requested info. I am assuming you do not actually need the street addresses of the institutions.

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Most instances of personal pronouns have been removed, however in some cases, e.g. Line 18 “We present a useful assay…” the personal pronoun is crucial for the sentence structure.

5. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials: e.g., MATLAB etc. We must maintain our scientific integrity and prevent the subsequent video from becoming a commercial advertisement.

References to MATLAB have been removed.

6. Line 91: Please ensure the unit for thickness is complete.

Line 91 on the manuscript proof “space between neighboring wells to ensure a good seal -- we found 3 mm to be” does not contain a unit for thickness. If the reviewer is referring to line 88 “Cut a piece of 250 µm thick silicone spacer sheet”, we believe this unit is complete. Is it possible that the Greek letter mu failed to display properly on the reviewer’s copy?

7. Line 97: For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks.

Time units have been changed as suggested.

8. Line 99/103: For SI units, please use standard abbreviations when the unit is preceded by a numeral. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm2.

Line 99 “over the wells starting from one edge” and line 103 “Place the sample onto the microscope stage and set magnification to the lowest” do not contain any mention of units. We’ve also checked around these two lines, and note that there are multiple uses of microliters, indicated in the manuscript as “µL”. We believe this is already consistent with the reviewer’s request.

9. Line 106-115: Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

The protocol step: "Acquire a clear image of a calibration slide or a clear ruler for pixel to millimeter conversion. " has been expanded to better explain as well as include an example calculation.

10. Please include a one line space between each protocol step and highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed. Thank you for the reminder, this has been fixed.

11. Please discuss all figures (Figure 3, Supplementary Figure 1) in the Representative Results. However, for figures showing the experimental set-up, please reference them in the Protocol.

Thanks for catching this omission. Both Figures 3 and S1 are now explicitly referenced in Discussion; "Specifically for the *S. coeruleus* population investigated here, their preference to settle in colonies (Figure 3) caused some of these cells not to be identified by the script." and "Supplemental Figure S1 provides these plots for the full timelapse, with identical axes used across all plots to give a clearer view of the expected increase of motility in a successful experiment. "

12. Figure 1: Please ensure that all the figures in the panel are labelled and the respective descriptions are in the Figure Legends.

Fig. 1 We've relabeled the figure so that all images have a respective label. This was checked with the figure caption to ensure they match. Labels A and B were correct while the originally labeled C was relabeled C-F and change D was changed to G.

13. Figure 3: Please include scale bars in all the images of the panel.

Fig 3. added scale bars to each image.

14. Supplementary figure S1: Please label each graph in the panel with respect to the hours after initiation of oral apparatus regeneration.

Fig. S1 labeled each graph with hours after regeneration.

Reviewers' comments:

Reviewer #1:

Major Concerns:

(a) While the authors provide graphs and images with quantitative information, it is a major concern that the authors due not give details on the quantitative analysis, especially the statistics in the text. The authors should give details regarding average

distance, , travelled and average velocity. The standard deviations should be used to show if variations are statistically significant. This is critical in demonstrating that this is a valid method to evaluate OA regeneration. Without this analysis, the manuscript should not be published since the validity of this method has not been backed by scientific evidence.

The representative results section of the manuscript has been rewritten to include a table of the mean and standard deviation of swim speeds, as well as t-test analysis demonstrating that the swim speeds between Phases 1 and 2 as and between Phases 2 and 3 are statistically significant.

(b) The discussion is essentially an extension of the protocol. Need a discussion of the results regarding validity, limitations, quantitative comparison to other methods and software, as well as future work.

The discussion has been edited to include a brief mention to other software. Validity of results presented has been addressed with the t-tests added into the representative results section. As JoVE is a methods journal, we intentionally avoided the discussion of future work and focused the discussion strictly on the method itself. The results presented are only to illustrate one application of this method.

Minor Concerns:

Below are specific suggestions

a) The authors are trying to introduce a new method of observing the effects of Oral Apparatus regeneration - the title should reflect that.

The title of the manuscript has been changed to "Analysis of Motility Patterns of Stentor During and After Oral Apparatus Regeneration Using Cell Tracking"

b) In the Abstract: insert "be" between "not" and "associated"

This change has been made.

c) In the Abstract and Summary: Replace "OA" with Oral Apparatus

The grammar and references in the abstract have been changed as suggested.

d) Summary: Give specifics on visualization method and indicate purpose of your manuscript.

The first sentence of the Summary has been changed to "We present a protocol for characterization of motility and behavior of a population of cells using brightfield microscopy and cell tracking."

e) How does the authors' tracking software differ from other video tracking softwares? Is there a special advantage? Cost? Run time?

The following text has been added to Discussion to address this. It is pasted here for ease of reference:

Many particle and cell tracking algorithms currently exist, some entirely free. Cost and user friendliness are often trade-offs requiring compromise. Additionally, After testing many of

these options, the protocol presented here is intended to be a one-stop solution to go all the way from data acquisition to data visualization using only low-cost equipment and a single scientific software package (Table of Materials). This is of particular interest to research labs at teaching focused institutions or biophysics teaching labs where most of the needed equipment is already available as it lowers the barrier for students to ask their own questions and arrive at quantitative results within a short timeframe.

f) Line 22: delete "here"

g) Line 53: Replace "would be to use" with "is"

h) Line 58: Replace "can not" with "cannot"

i) Line 58: Replace "done" with " achieved by"

j) Line 91, 99 and 103: Unit is not properly displaying in pdf file.

Thank you for the suggestions items f through j, they have all been incorporated into the revised manuscript.

k) Line 112: insert "the" before "light" and be more specific about what light you are referring to.

The text has been changed to "Avoid leaving the sample illuminated when not imaging".

l) Line 128: Refer to figure of cell traces

A figure showing cell traces is now referenced later in the protocol.

m) Line 141: "See Discussion" should be lower case

This revision has been made.

n) Line 154: refer to figure showing traces

Line 154 describes linking beginning-of-video cells with end-of-video cells to check the accuracy of traces, but the figures in the manuscript only show the completed traces, not this color-coordinated linkage. Figure 1B is now referenced a few lines later, where the tracing process is complete.

o) Line 170: What kind of plot? Specify

Specified as follows: "one radial displacement versus time plot for each timepoint"

p) Line 185: Refer to figure illustrating your point

A figure showing combined traces has been referenced.

q) Line 187: replace "up to the" with "a". Replace "equals" with "of up to". Delete circle.

These revisions have been made.

r) Representative Results: 1. Refer to the evidence in your figures. 2. Present quantitative statistics with standard deviation. 3. How does the average speed change over time? 4. It might be interesting to determine in future work if "directionless" swimming. Is truly directionless/random and what is the dynamics during the transition. You can test that with lag plots.

T-test results have been added to the Representative Results section. Average speeds have been added to the same section in new table under "mean". Thank you for the

excellent suggestion of lag plots for the directionless traces. We suspect there is a constant curvature to the motion and this should indeed show up in a lag plot.

s) The font color is mismatched throughout the document between gray and black.

t) Line 231: spell out "MB"

The font color has been fixed, and “membranellar band” has been spelled out.

u) Phase 1: Give quantitative results. Give standard deviation (SD) to indicate spread.

Standard deviations have been included in new table in Representative Results.

v) Line 250: Incomplete sentence

The sentence has been revised.

w) Line 258: By how much do speeds drop on average? SD

Standard deviations have been included in new table in Representative Results.

x) Line 263: Give SD to back up your claim of statistical significance.

Standard deviations have been included in new table in Representative Results.

y) Line 275: Say what these results are and why they suggest full regeneration. Back this up quantitatively.

We agree that the original statement was vague. The corresponding sentence in the revised manuscript now reads: “The lack of statistically significant difference between hours 8-10 (Phase 3) and hours 11+ (Phase 4) suggest that the cells have fully regenerated by this time and few genes should be differentially regulated beyond hour nine.”

z) Figure Captions: fig 1: Letters do not match figure.

Fig. 1 We’ve relabeled the figure so that all images have a respective label. This was checked with the figure caption to ensure they match. Labels A and B were correct while the originally labeled C was relabeled C-F and change D was changed to G.

The font is small and somewhat grainy in part C of fig. Suggest enlarging font.

Fig1 C increased text size for both title and axis. Made figures overall more clear.

fig2: Give details in the text about your normalized counts: total number of cells.

This has been clarified in the caption to Figure 2 with the addition of the following sentence “Multiple datasets which span different subsets of hours were combined for this figure, so total cell count per hour ranged from 57 to over 376. “

Is there an uncertainty when some cells are in a hybrid stage? italicize or put in quotes "imshowpair." Explain in the text how "imshowpair" works; and how well it works. Points in B are grainy.

Fixed all figures to make them more clear.

State results in caption; i.e., average speeds.

The means and standard deviations of data presented in Figure 2 are now summarized in a table in Representative Results.

Line 294/5 article "the" missing twice.

The sentences have been revised.

fig. 3: It looks like there are no tracks in A and very little tracks in B. Maybe the images got mixed up?

Fig. 3 the images did get mixed up. Changed the figure caption to properly label the images of A, B at 12 hours and C, D at 18 hours.

Yes, there is an uncertainty for the cell counts in Figure 2 on whether a holdfast (tail-like part of a healthy Stentor) is visible or not. Partially regenerated cells have small holdfasts which can be obscured if a cell is oriented away or against a wall. This figure is included because to our knowledge such a count of motile vs. non-motile cells through the time course of Stentor OA regeneration does not currently exist in literature.

S1: Replace "77" with "7."

Thanks for the catch, the 77 was fixed.

aa) Figures: Identify/label air bubbles in images.

Changes made for Fig. 1 B and Fig. 3. Added transparent, black striped circles to indicate air bubbles in images.

bb) Give average speeds at different stages. Is the difference statistically significant? If they are not, say so.

Table has been added into Representative Results section with this info.

cc) Discussion: It look like the entire discussion belongs into the protocol. The discussion is essentially an extension of the protocol. Need a discussion of the results regarding validity, limitations, quantitative comparison to other methods and software, as well as future work.

The discussion section has been edited to improve clarity. We intentionally avoided mention of future work as JoVE is a methods journal.

Explicit statistical analysis (t-tests) have been added to the Representative Results section to demonstrate validity.

An opening paragraph has been added to the Discussion which addresses " quantitative comparison to other methods and software"

Many particle and cell tracking algorithms currently exist, some entirely free. Cost and user friendliness are often trade-offs requiring compromise. Additionally, after testing many of these options, the protocol presented here is intended to be a one-stop solution to go all the way from data acquisition to data visualization using only low-cost equipment and a single scientific software package (Table of Materials). This is of particular interest to research labs at teaching focused institutions or biophysics teaching labs where most of the needed equipment is already available as it lowers the barrier for students to ask their own questions and arrive to

quantitative results within a short timeframe.
dd) Line 312: An illustration would be helpful here.
Supplemental Figure 1 shows motility patterns at timepoints throughout the regeneration process.
ee) Line 346: Explain "MOB1"
During the revision process it became clear this explicit mention of MOB1 is not necessary, so it has been removed. MOB1 is a genetic phenotype of Stentor with altered regeneration and morphology, as discussed in reference #6.
ff) figures: Include scale in microscopic images and tracks.
Fig 3. added scale bars to each image.
fig 1C: The 2D Fourier Transforms will help with quantifying the spread of tracks. And/or you can calculate the traveled.
We thank the reviewer for the suggestion of 2D Fourier Transforms. This is a technique we will explore with future datasets where we screen for motility phenotypes. Within the scope of this JoVE protocol, the script SpaghettiPlots.m which generates the plots in Fig1C is intended as a quick, qualitative visual comparison.
S1: Put respective hours on images A-M.
Fig. S1 Labeled each image with respective hours after regeneration.

Reviewer #2:

As is, the manuscript is somewhat too focused on one issue only (oral apparatus regeneration). However, in fact the assay and analyses can be applied to address many questions that involve Stentor motility. So I wonder whether the manuscript can be made more relevant/interesting to a broader readership by reducing the focus on oral apparatus regeneration. In fact, the authors themselves say in the summary "We present a useful assay and data visualization method for characterization of motility and behavior of a population of cells."
We agree that as written, the manuscript is a bit too focused on the specific application to OA regeneration of Stentor and that it will be more broadly useful to researchers looking to apply it to other biological systems if more focus is placed on the protocol and the statistics which come out of the analysis. We have shortened the discussion which sought to contextualize the results within the literature on Stentor and added discussion on the statistical analysis. The part of the discussion on how to adapt the protocol to other cell types has also been edited for better readability.

Examples:
In 1.4: The authors write "be careful of matching the well volume as closely as possible." What is being matched to the well volume?

Sentence clarified to: “Be careful of matching sample volume to the well volume…”

In 2.1: What magnification(s) would be useful? Here the reason is given (fit one well), but no values are suggested.

The sentence “A 1.6x camera adaptor and 1x magnification on the objective were used on our microscope” has been added.

In 2.2: Why are the videos 10 s, not more and not less? What is the frame rate of those 10 s videos? Also, in a timelapse, what is the time between frames? I don't think this is mentioned anywhere.

These parameters were not specified because they are not critical. The video needs to be long enough to capture motility patterns and acquired at high enough framerates not to miss any sudden changes in motion. For S. coeruleus, 10s of video acquired at 30fps was good.

In 3.8: There are tracking parameters, but they are not explained or specified anywhere. The reader is referred to the discussion, but in the discussion section there is little discussion of the tracking parameters. There is some discussion about cell detection, but nothing about tracking. A table of parameters (detection, tracking, anything else), their meaning and values, will be helpful.

We agree that detail was lacking here. The following text has been added into the manuscript: “Several parameters in the TrackCells.m script can be tweaked to optimize the automatic cell identification and trace validation. The parameters MinCellArea and MaxCellArea (Lines 15 and 16 of script) allow the user to set the acceptable range of sizes for a cell, in square pixels. What values are best depends on many details of the experiment including cell size, camera pixel size, magnification, and whether there are objects which could be misidentified as cells, for example air bubbles. The default values of 300 and 1500 were optimal for the exact equipment and settings provided in the protocol. After MinCellArea and MaxCellArea have been optimized, tune the parameter Threshold (Line 17) to change image contrast. When incorrectly set, the cell outlines will be poorly identified or missed altogether, thereby preventing the tracking to be done correctly. If no value of Threshold works well for correct tracking, try with a video with higher contrast or is more in focus. Line 218 of the script, “bad_trks = find(strk_trks > 20);” is responsible for discarding traces which deviate from the predicted motion (via Kalman filter) too many times. As is, the script has this threshold for “too many” set at 20. Decrease this integer value down, as far as 1, to discard traces more aggressively. Increase the value to discard less aggressively. “

In 4.4: It is not clear from what is written how the in-between points of a trace are validated.

The trace validation algorithm is based on the Kalman filter, which assumes Gaussian noise and predicts the next point in a trace based on a joint probability distribution

constructed from prior points in the trace. This is an established algorithm used in signal processing.
In 4.6: Why stop at 60? Also, can one fix traces, or only accept or reject them?
As typically there will only be around 10 cells in one well, and ideally there should only be one continuous trace per cell identified by the script, 60 was chosen as a safe upper bound. The current script does not allow for fixing traces. It is possible to do this by going into the variable where the traces are stored and manually copy/paste two traces together. We did not include this info in the protocol as it is intended to be for an audience which is not familiar/comfortable with code.
Line 204: "Y" hours???
Changed typo of “Y hours” to “7 hours”.
Line 302: I assume 7, not 77.
Thanks for the catch, line 302 has been fixed.

The writing must be cleaned up. There is quite some confusion of plural and singular and present tense and past tense, e.g. "Cells display little to no visible motion and visually lacks morphology" and "The range of movement of the most motile cells in the population gradually increase during this phase, while the cell speeds were surprisingly varied" (starting with line 247). I emphasize there are multiple such grammatical mistakes; these are not the only ones.
The grammar and writing throughout the manuscript has been proofread and revised, and the specific suggestions have been implemented.
In a few places, a Greek symbol (probably "micro") does not appear properly on my computer (all I see is a rectangle with a question mark).
The “micro” symbol (μ) was the one not displaying correctly, it has been switched with a version that should display on most computers.

Reviewer #3:

Major Concerns:
The figures are poorly visible (resolution problem), and are not all referenced in the text (Figure 3, and Figure S1).
Increased text size and fixed all figures so that they are more clear.
Also some figure panels do not exist (figure 1 E-H).
Another panel was added to Figure 1, and in-text references were changed to match the figure.
We recommend the following changes:

- The authors should add a figure panel displaying a simplified representation of S. coeruleus regeneration steps.

The figure panel displaying the regeneration steps has been added to Figure 2, it is now Figure 2A.

- Figure 1C should include the n number of traces represented on each panel. Also the scale should be similar on each panel for clarity reasons.

Fig. 1C Added n number of traces to each image. However, the purpose of this figure is to show the way the cells are swimming and their change over time. In order to show this, the figures must be zoomed in on the traces.

- On Figure 2, a clear explanation of what the holdfast is/looks should be displayed. The representative images of each population should be blow up and contain accurate legends.

A drawn figure and explanation of the holdfast is addressed in figure 1.

- Figure 3 and 3 are hard to understand by lack of explanation and not mentioned in the text.

Figure 3 is now explicitly referenced in the Discussion as follows: "Specifically for the *S. coeruleus* population investigated here, their preference to settle in colonies (Figure 3) caused some of these cells not to be identified by the script." We hope this clarifies the intention behind the Figure.

Parts of the Representative Results section should re-written or modified:

- Figures are not always referenced at the correct location in the text. e.g: after "while at nine hour many cells traversed more than …" Figure 1C should be referenced.

Figure 1C is now referenced in the text preceding this line, and other references to figures have been added throughout the text.

- Style should be improved in that section. Notably, I recommend re-writing the part describing the phases of movements in the style of the scientific articles, avoiding subsections. Also figures should be referenced in the text as (Figure X) and not among sentences (e.g. "An example of this can be seen in the final inset of Figure 2B"). Also references should be cited at the proper location, and not at the beginning of the section (e.g "….. from the work of Sood et al., and Onsbring et al." : these references should be implemented after each statement related to them)

The section has been rewritten and shortened.

- The authors should define the term holdfast for non-specialists

Figure 1 has been modified with additional illustrations to clarify the morphology of Stentor and an unnecessary reference to the holdfast has been removed from the Discussion.

- The authors should discuss in the Result section the differences seen between speed and movement type, and why the two parameters do not always correlate.

Speed in the manuscript refers to instantaneous speed of a cell as averaged over an entire trace. The directionless cells during Phase 1 have lower speed than regenerated and motile cells in Phases 3 and 4. This difference is not apparent in the mean because the morphologically healthy Stentors form small colonies of stationary cells, thereby lowering the mean during Phases 3 and 4.

- In the Phase 3 part, the authors should indicate which statistical test allowed them to find significance, either in the text, either in the figure legends. The authors should relate the statement about kinases kinesin etc to the observation more clearly.

Details on the statistical tests have been added to Representative Results. The statement about kinases etc. has been removed from the manuscript as part of the edits to improve clarity to that section of the manuscript.

- A paragraph of conclusion and perspectives should be added to this part (for example, the last paragraph of the discussion).

Please clarify which part? An opening paragraph has been added to the Discussion which may serve the same purpose (pasted below):

Many particle and cell tracking algorithms currently exist, some entirely free. Cost and user friendliness are often trade-offs requiring compromise. Additionally, After testing many of these options, the protocol presented here is intended to be a one-stop solution to go all the way from data acquisition to data visualization using only low-cost equipment and a single scientific software package (Table of Materials). This is of particular interest to research labs at teaching focused institutions or biophysics teaching labs where most of the needed equipment is already available as it lowers the barrier for students to ask their own questions and arrive to quantitative results within a short timeframe.

We recommend that the discussion section is renamed as a troubleshooting and integrated to the Protocol section, as it is more relevant to it. A discussion section should be related to the representative results. We leave at the discretion of authors to chose writing a separate discussion section, or to discuss the results straight in the representative result section (the second option has our preference).

The discussion section has been edited to improve clarity. We respectfully disagree with the reviewer's suggestion to make the discussion section more "related to the representative results", as this is contrary to the guidance provided by the guidance in Information for Authors, which states "JoVE is a methods journal. Thus, the Discussion section of the article should be focused on the Protocol."

The authors should provide sample videos in the script package for the reader to be able to test the script and see typical results.

Thank you for this suggestion, we will include 3 sample data videos for readers to confirm the scripts are working.

Minor Concerns:

The summary/abstract are well-written, and the authors clearly present the usefulness of their protocol. A couple remarks:

- The authors should specify clearly in the summary to which types of cells/organisms the protocol can be used for.

The summary now specifies "a population of hundred micron to millimeter sized cells"

- Any Matlab alternatives ? It is acceptable if not, but maybe could be stated earlier in the paper.

JoVE requires specifics about materials (which the software falls under) to be listed in a separate Table of Materials. There we specified that the software necessary is an installation of MATLAB with the Image Processing Toolbox and the Statistics and Machine Learning Toolbox.

The Protocol section is clear and reads well. Some comments:

- the micrometer symbol appears as an exclamation point in the manuscript

This has been fixed.

- A figure/video should be added for different steps of the protocol if possible. In particular parts 1 and 2.

As part of the revisions, most of parts 1 and 2 of the protocol have been highlighted to designate them as parts to be filmed for the video protocol part of this JoVE submission.

- the authors should indicate what is the maximal concentration of S. Coeruleus they recommend for this protocol.

As specified in Protocol step 1.4, we recommend "approximately ten stentor into each well", and the wells are 5/16" in diameter (step 1.2).

-The authors should indicate in the text the type of microscope used for this protocol. They should also indicate the optimal ocular used for acquisition.

The exact model of the microscope (Leica S9E and AxioZoom A.16) are specified in accompanying Table of Materials. Additionally, Protocol step 2.1 now states "A 1.6x camera adaptor and 1x magnification on the objective were used on our microscope."

- The authors should indicate the optimal frequency of acquisition (every second ? every 500 ms ?)

Step 2.2 of the protocol has been edited to state "Acquire a ten second video at 30 frames per second of each well at each timepoint."

- Notes should not be unique protocol steps (e.g. 2.3)

Notes have been incorporated into the appropriate protocol steps as recommended.

- Is it possible to use the pixel size of the camera instead of the ruler ?

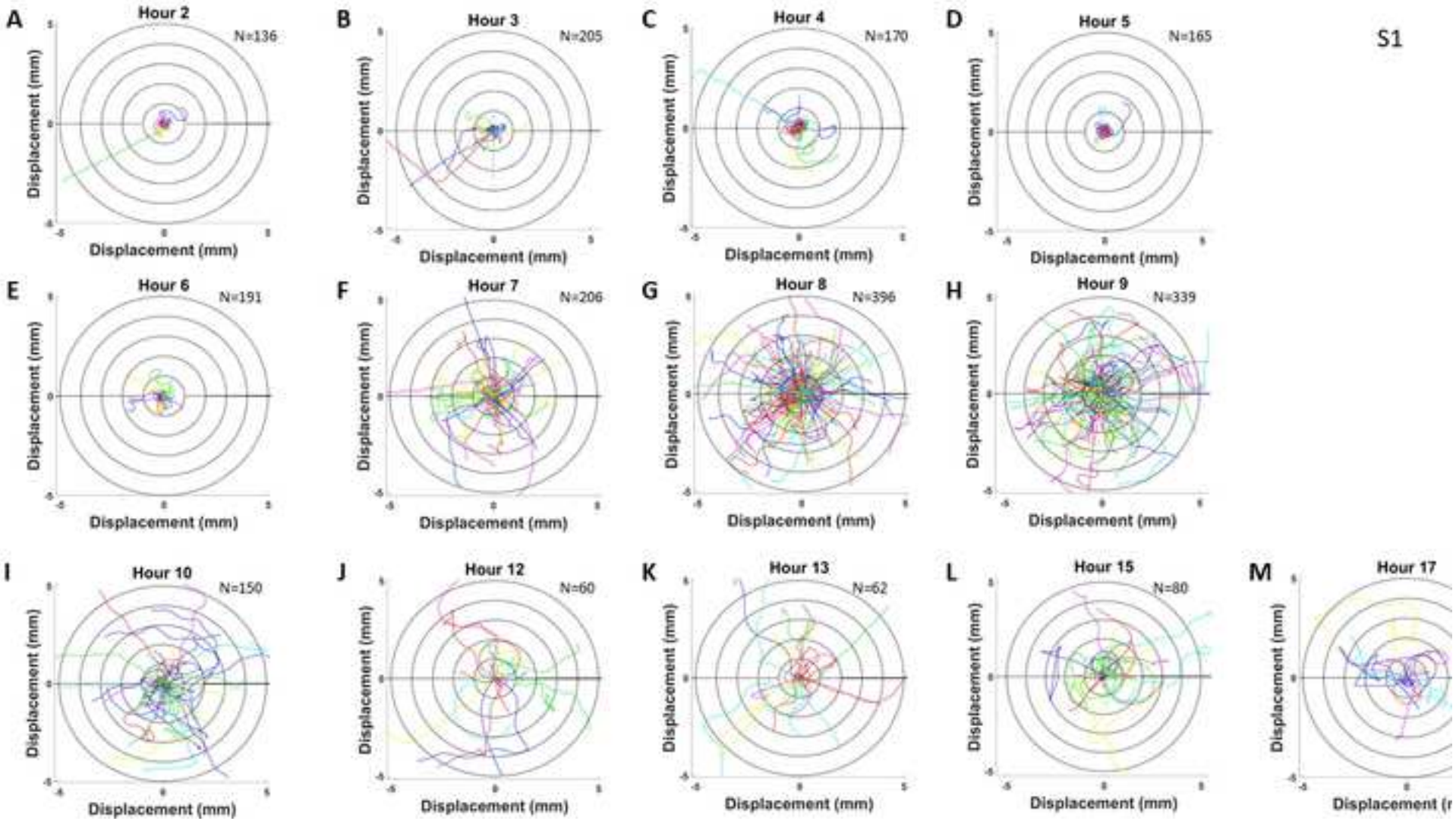
A wide range of camera pixel sizes are suitable for this protocol, as long as they are paired with a suitable magnification setting on the microscope. For our data, the camera pixel size was 3.45 μm and the magnification used was 1.6x.

- The authors should specify that scripts needs to be added to the Matlab path prior to using.

Step 3.4 has been added: “Choose “Add to Path” if prompted by a pop-up window. This typically only happens when the script is run for the first time from a given folder. “

-The authors should indicate the configuration recommended for running their scripts.

As indicated in the Table of Materials, MATLAB with the Image Processing Toolbox and the Statistics and Machine Learning Toolbox are necessary for running the scripts. We have also added to the Table a recommendation for a computer with at least 4GB RAM.



S1

Sample data Supplemental video 1

Sample data Supplemental video 2

Sample data Supplemental video 3

Click here to access/download
Supplemental Coding Files
2018_01_19_99_14.MOV