# TumbleScore: Run and tumble analysis for low frame-rate motility videos

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Scientists often exploit the motility of peritrichously flagellated bacteria for various applications. A common alteration is modifying the frequency of mid-movement changes in direction, known as tumbles. Such differences in bacterial swimming patterns can prove difficult to quantify, especially for those without access to high-speed optical equipment. Traditionally, scientists have resorted to less accurate techniques, such as soft agar plate assays, or have been forced to invest in costly equipment. Here, we present TumbleScore, software designed to track and quantify bacterial movies with slow, as well as fast, frame-rates. Developed and fully contained within MATLAB, TumbleScore processes motility videos and returns pertinent tumbling metrics, including: (*i*) linear speed, (*ii*) rotational speed, (*iii*) percentage of angle changes below a given threshold, and (*iv*) ratio of total path length to Euclidian distance, or arc-chord ratio (ACR). In addition, TumbleScore produces a "rose graph" visualization of bacterial paths. The software was validated using both fabricated and experimental motility videos.

Bacterial motility has been of great interest to researchers and clinicians for decades (1). Motility phenotypes have been characterized using methods as disparate as soft agar plate assays (for swimming and swarming) (2,3) or 3-D high-resolution tracking of single cells on a translational microstage (4). The latter provides precise quantitative analysis of bacterial movement in 3-D, including rotational analysis as well as X, Y, Z displacement. The former is far simpler, but the results are less reliable as they depend greatly on nutrient availability, agar quantity, humidity, temperature, and many other factors. Notwithstanding this variability, agar plate assays remain a method of choice due to their relatively low cost, minimal laboratory equipment requirements, and facile quantification.

Tracking microscopy requires synchronized high-speed optical equipment, a significant amount of data storage, and large-scale data manipulation capabilities. While the results are far superior to soft agar plate assays, the method is not as widespread.

Here, we present a rapid, inexpensive methodology for tracking bacterial swimming using simple bright-field and fluorescence microscopy coupled with low-speed video capture. Once bacterial paths have been ascertained, analysis tools can be used to quantify complex motility behaviors. Data can be manipulated in spreadsheets and with MATLAB. Results are quantitatively precise and statistically validated. Moreover, the resultant phenotype is indeed free swimming in quiescent and low-velocity flows.

The motility behaviors of peritrichous bacteria, such as *Escherichia coli* and *Salmonella typhimurium*, are well documented (1,5–8). In response to a chemical attractant, repellent, or neither, the organism controls its movements by modulating flagellar rotation (9). As depicted in Figure 1, when bacterial flagella turn counterclockwise, they form a bundle and push the cell in a relatively smooth path, called a "run." Occasionally, the flagella will turn in a clockwise direction, disbanding the bundle and causing the cell to change direction randomly and rapidly while stopping forward motion. This motion is called a "tumble" (10). In *E. coli*, the balance of intracellular chemotaxis Che motility regulator proteins controls the pattern of flagellar rotation and, thus, the tumble frequency (8). For example, a low CheZ:CheY ratio results in a high tumble frequency and vice versa (11). Tumble frequency is inversely related to directionality (i.e., a cell's propensity to travel

## METHOD SUMMARY

TumbleScore is a MATLAB-based software for processesing bacterial motility movies to obtain swimming behavior metrics. The program automatically segments video frames, identifies cells, tracks movements, removes noise, and performs specialized analyses.

in the same direction for an extended period without interruption). Characteristics of low-directionality swimming include: (*i*) high path tortuosity, (*ii*) low Euclidean distance (displacement), (*iii*) high rotational speed, and (*iv*) low linear speed (12). Tumble frequency is difficult to assess because assessment depends on the ability to identify individual tumble events. As an alternative, researchers make observations of directionality and align these with the tumble frequency (13).

We and others (14,15) have developed several applications that exploit swimming and motility functions of bacteria, in particular as a means to deliver therapeutic payloads synthesized by the swimming cells (16,17). We have exploited *che* mutants (which do not run) to directionally guide cells to specific sites, a phenomenon referred to as pseudotaxis (18,19,20). While directionality en masse has proven sufficient, it is highly desirable to develop a detailed understanding of the performance of cells specifically engineered to provide programmed motility. Thus, it has become necessary to closely analyze bacterial tracks under these conditions.

While the resultant phenotypes are obvious to the naked eye under simple bright-field microscopy, and manual analyses can be employed to provide details of swimming speed and even tumbling from bacterial motility videos, such methods are laborious and, more importantly, prone to bias. Automated cell tracking software relieves both time and bias by providing a quicker and more objective analysis of videos. Current commercially available programs can provide the requisite linear speed information, but rarely provide other indicators of directionality.

Linear speed is an effective yet somewhat incomplete indicator of directionality. A more explicit metric for tumbling is often desired. One approach involves the calculation of instantaneous velocities and rate of change of direction (RCD) between every frame of an individual cell trajectory. A synchronous decrease in linear speed and increase in RCD over a number of frames signifies a tumble (21,22). This method relies on cameras with high temporal resolution—enough to capture multiple images of a single tumble. The tumble duration

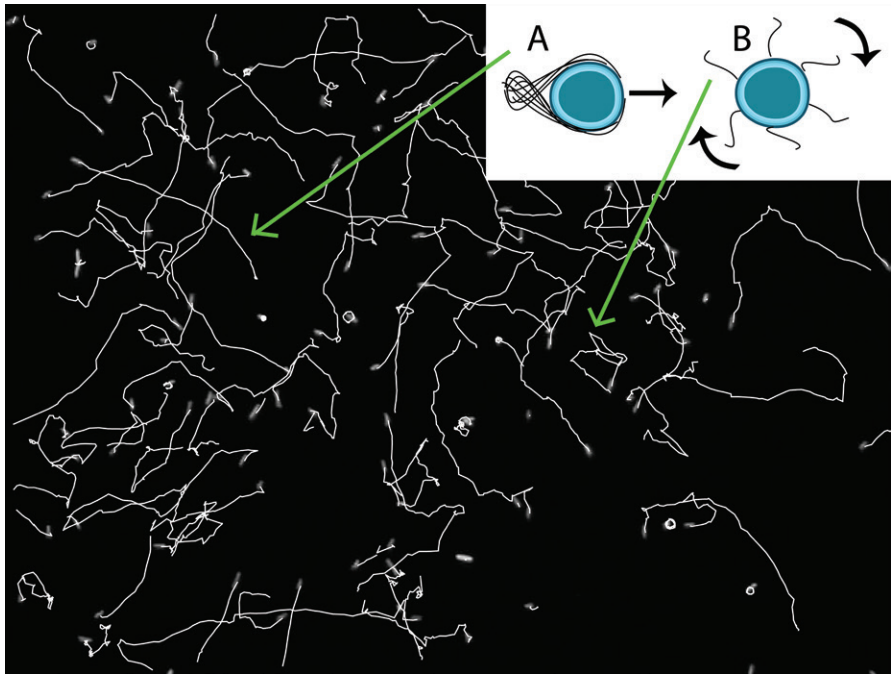


**Figure 1. Bimodal swimming of peritrichously flagellated bacteria, shown both in a cartoon and in actual output from TumbleScore.** Trajectories are of wild-type (WT) *E. coli* cells. TumbleScore output video can be found in Supplementary Video S1. (A) When the flagella are rotated in a counterclockwise fashion, they form a bundle, and the bacterium moves forward in a linear fashion. The arrow from (A) points to a trajectory that has a high run duration. (B) When the flagella are rotated in a clockwise fashion, they disperse; the bacterium performs a random tumble and changes direction. The arrow from B points to a trajectory that has a high tumble frequency.

for *E. coli* is known to vary (e.g., 0.20 ± 0.05 s reported in Reference 23 and 0.10 ± 0.05 s reported in Reference 6). As such, a video frame rate slower than ~0.1 s frame$^{-1}$ prohibits capture of crucial information about instantaneous linear speed and angle changes throughout a tumble. At a slow frame rate, two subsequent frames can only capture a cell's transition from run-to-run, run-to-tumble, and tumble-to-run.

Even with this limited information, however, it is possible to quantify directionality beyond linear speed. Providing metrics for rotational speed and path tortuosity are effective ways to do this (12). A cell will transition from run-to-tumble and tumble-to-run on a more frequent basis when tumbling is high, causing a higher RCD. In addition, a greater percentage of angle changes (%S) will be smaller than an arbitrary "smooth threshold" angle. Previous studies have determined that rotational speeds <1 rad/s can be associated with a run, while those >3 rad/s can be associated with a tumble (23). Path tortuosity, a product of how frequently a bacterium changes direction, can be demonstrated with the ratio of total path length to net displacement—the arc-chord ratio (ACR). A cell that travels in a straight line will have an ACR of 1, while a cell that spins in place will have an infinite ACR.

Semisolid agar plates are used to visualize the phenomenon of low net displacement in cells with a high tumble frequency. A similar pictorial can be digitally attained post-processing by translating cell trajectories to begin at a common origin point and sketching their movements outward for a set length of time. Because of their shape, we refer to these figures as "rose graphs." As tumbling is reduced in a sample, one can see the rose bloom.

TumbleScore, a MATLAB tool, automatically tracks and reports cell tumbling behavior, providing the metrics discussed above: linear speed, RCD, %S, and ACR—as well as various visual accompaniments. TumbleScore data can immediately be used for analysis and publication.

We have built TumbleScore with only a minimal MATLAB GUI to provide both maximum user-friendliness and flexibility. To use the software, users should load it into MATLAB, provide the motility video in the proper format, supply basic infor-

mation such as frame rate, pixel-to-nm conversion, etc., select analysis options, and choose which outputs they want. Users may choose to vary only the few parameters required or, conversely, may interact directly with the source code. Additionally, users may combine data from multiple videos by specifying the number of videos before analyzing them. The program and complete instructions on its use are freely available at www.bioe.umd.edu/bentley.

## Materials and methods

### Strains and growth conditions

The K-12 W3110 *E. coli* strain was used for all experimental videos. Cells were grown in LB media supplemented with appropriate antibiotics at 50 μg/mL. pFZY1 plasmids conferred ampicillin resistance, and the pET vectors used in this study conferred kanamycin resistance. Cells were grown at 37°C, with shaking at 250 rpm. *E. coli* K-12 W3110 Δ*cheZ* cells were used for motility negative controls. Also, Δ*cheZ*- cells were those transformed with pFZY1 to overproduce CheZ based on a peroxide chemical inducer, $H_2O_2$. All cells were also transformed with *pT5RT7G*, which confers constitutive *egfp* expression (20,24). These cells are thus very bright when imaged using fluorescence microscopy, allowing for superior contrast.

### Motility videos and analysis

Overnight cultures were reinoculated in fresh LB medium and grown to an $OD_{600}$ of ~0.45, and inducer was added to the appropriate samples. Cultures were grown at 30°C and sampled by withdrawing small volumes (100 μL) and centrifuging (400 × *g*) for 5 min. They were then resuspended in an equal volume of chemotaxis buffer (CB) (1 × PBS, 0.1 mM EDTA, 0.01 mM L-methionine, 10 mM D,L-lactate). A 2-μL droplet was placed on a microscope slide rinsed with CB, and a coverslip was placed over the cells. Cells were recorded using CellSens software and a DX60 microscope equipped with a DP72 camera (Olympus, Waltham, MA). Approximately 100 frames were recorded for each video using a 20× objective lens with a 125 ms exposure. The videos were transferred into JPEG image sequences using FIJI (www.fiji.sc).

TumbleScore was written and tested in MATLAB version 2014b (The MathWorks, Natick, MA), although it may also work in previous versions. The MATLAB Image Processing Toolbox is required in order to use TumbleScore.

### Object tracking

Video images were first converted into a grayscale matrix, with matrix indices representing pixel intensity as an integer between 0 (black) and 255 (white). The image was then segmented using a user-submitted pixel intensity threshold or Otsu's method of thresholding, which uses a histogram method to differentiate between bright and dark pixels, and each frame was converted into a binary matrix (25). This method provides reproducibility across videos. TumbleScore allows users to test object detection before running the program.

In order to localize cells in a given frame, TumbleScore utilizes *regionprops*, a built-in MATLAB function. This function uses edge detection to segment the image into regions (i.e., cells). Furthermore, *regionprops* provides relevant information for each cell, including: centroid location, area, perimeter, major axis length, orientation, and maximum pixel intensity. Cells partly out of frame are ignored.

A "nearest-neighbor" approach is used to link cells from adjacent frames into trajectories. Cells in the first frame are added to a list of trajectories. Cells in the subsequent frame that are within a user-submitted distance, are identified as "suitors." Suitors are compared by area, perimeter, length, and maximum pixel intensity to the trajectory cell in the previous frame and ranked by property similarity. Maximum pixel intensity is considered because it corresponds to cell width and position in the *z*-plane. Because of the asymmetrical nature of *E. coli* motility, previous trajectory speed or direction is not considered when linking suitors to trajectories. For the case where two suitors are equally similar to the trajectory cell, the suitor closer in distance is favored. Before matching a suitor to a trajectory, the suitor must be tested against all as-of-yet unmatched nearby cells in the previous frame. If there is no closer match for both trajectory and suitor, a match is made and the trajectory extended. If no match is made for a trajectory, the trajectory is ended. If no match is made for a suitor, a new trajectory begins. Any trajectory that ends with fewer frames than a user-submitted minimum is discarded. TumbleScore outputs a video identical to the original, except with trajectory path lines overlaid (see Figure 1).

In Figure 1, calculated trajectories of approximately 100 cells are presented. Once trajectories are specified, parameters associated with swimming are presented. A trajectory's linear speed is the total path length divided by the

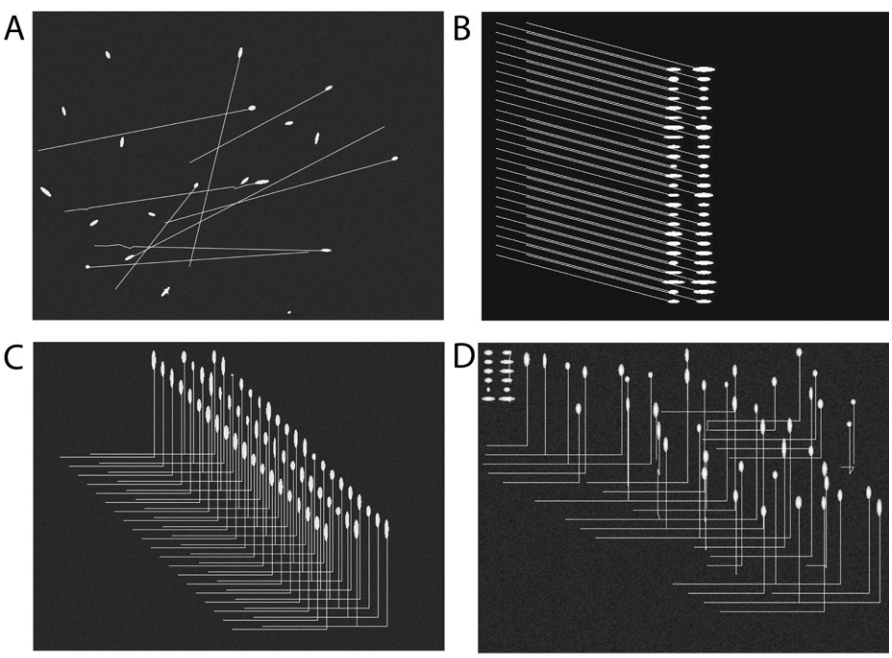


**Figure 2. Final frames of control videos.** Control videos tested (A) linear speed, (B) rate of change of direction (RCD), (C) arc-chord ratio (ACR), and (D) a more realistic test.

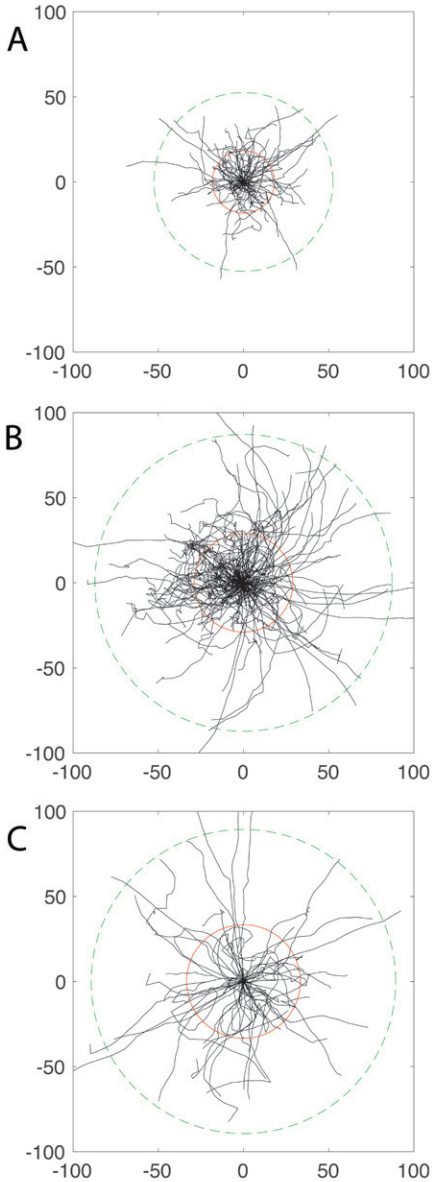


**Figure 3. Portfolio of rose graphs from experimental videos.** Bacterial cells lacking the CheZ protein will have a higher tumble frequency and, thus, an overall lower net displacement, or "spread." (A) W3110 Δ*cheZ* cells have a relatively low spread. (B) Wild-type (WT) W3110 cells, which naturally express CheZ, have a high spread. (C) W3110 Δ*cheZ* pFZY1 cells, with restored CheZ production, exhibit a high spread similar to that of WT cells. Distance units are expressed as micrometers (μm). All trajectories were generated from 5 s of each trajectory. Circles indicate the mean and 90th percentile net displacements.

trajectory time. We do not account for movement in the *z*-plane; this is an obvious limitation to the approach. That is, cells with significant *z*-component speed have trajectories that are eliminated based on their disjointed frame-by-frame linkages. Thus, our approach calculates swimming characteristics from freely swimming cells having trajectories largely in the *xy*-plane. Cells often leave the field of view, enter the field of view, move out of focus, or collide with other cells (which may lead to TumbleScore confusing the trajectories). Therefore, it is possible for a single cell to create multiple trajectories over the course of the video. For that reason, any results are reported as trajectory data, not on a per-cell basis. This is in line with the 2-D nature of our experimental set up and the focal plane of our microscope. Parameters calculated include: average population linear speed, standard deviation (SD), and standard error (SE). A histogram of trajectory velocities is produced.

## Removing noise

Because cells are at times stuck to the coverslip or microscope slide or there may be movement due to convective flow in certain microfluidic devices, we have developed methodologies to remove erroneous trajectories. Fixed cells are identified as those where the total cell displacement calculated at both half- and full-trajectory times are below a user-specified distance. These trajectories are then discarded. Additionally, some cells become stuck or unstuck midway through a video, or the trajectory of a moving cell becomes confounded with a cell that is fixed. To remedy this, if a cell repeatedly travels less than a user-submitted distance between frames for a user-submitted length of time, then that section of trajectory is discarded. It is important that the length of time is substantial or else the program may confuse a stuck cell from one that is tumbling. In addition, the program includes an option to remove outliers from video results, using a modified Thompson Tau method for a user-submitted significance level.

Because it is difficult to ensure that there is no fluid flow and it is equally difficult to account for flow perturbations that arise during video capture, we developed a filter for drift. The direction of movement for every trajectory is found for every pair of adjacent frames. The mode of the directions for each pair of frames is determined, and the "fluid velocity" is defined as the average speed of the trajectories that move in the mode direction for the greatest number of frames. For each trajectory, a ratio between the number of frames traveled with and against the mode direction is found. If the ratio is high and the trajectory linear speed is sufficiently near the fluid velocity, the trajectory is discarded. An example of this is shown in Supplementary Figure S1. This feature can be deactivated in any case where the user sees fit, such as if a low number of cells were imaged.

## Rose graph

In order to provide a visual companion to linear speed and directionality values in bar charts, TumbleScore takes a cue from the traditional motility plate. That is, trajectories from a single video are realigned so that the initial location is at the origin of the *xy*-plane. In this depiction, directional bias and net displacement are readily discerned. TumbleScore allows to users to include circles whose radii correspond to the mean and 90th percentile net displacements, as seen in Figure 3. The rose graph draws data from only one video, but users can certainly overlay image files.

## Tumble

TumbleScore uses *regionprops* to find cell orientation in each frame. By comparing the cell orientation in subsequent frames for every trajectory, TumbleScore provides average population RCD (in degrees per second), SD, and SE. For each trajectory, TumbleScore checks if the angle change between subsequent frames is smaller than the "smooth threshold," and finds the "smooth percentage," %S. TumbleScore provides the average population smooth percentage, along with the SD and SE. TumbleScore also provides the mean and median population ACRs, as well as the first and third quartiles for the ACR. The equation for ACR is

$$ACR = \frac{\sum_{i=1}^{n-1}\sqrt{\left(x_{i+1}-x_i\right)^2+\left(y_{i+1}-y_i\right)^2}}{\sqrt{\left(x_n-x_1\right)^2+\left(y_n-y_1\right)^2}}$$

where $(x,y)$ is the cell position, and $n$ is the number of frames. A rose graph displaying the total path and net displacement of trajectories where ACR ≈ 3 can be found in Supplementary Figure S2. Since these directionality approaches are potentially time-dependent, TumbleScore asks the user to specify a uniform length of time for the trajectories for which he or she would like tumbling analysis.

**Table 1. Comparing *E. coli* sample tumble frequencies.**

| Sample | Average ACR | Average speed (μm/s) |
|---|---|---|
| *W3110* Δ*cheZ* **(A)** | 3.33 | 8.66 |
| Wild-type (WT) W3110 **(B)** | 1.69 | 11.84 |
| *W3110* Δ*cheZ* pFZY1 **(C)** | 2.12 | 12.21 |

Tumble frequency represented by the arc-chord ratio (ACR) and linear speed. Samples deficient in CheZ (A) have a higher tumbling frequency, and therefore a greater ACR and lower speed. The normal frequency (B) is restored when CheZ expression is induced (C). ACR values were generated from first 5 s of each trajectory.

## Results and discussion

We developed a series of control experiments wherein source trajectories of known quantity were analyzed by TumbleScore. As input, we provided (*i*) trajectories of cells placed randomly in the original frame but moving in known straight paths; (*ii*) cells moving horizontally with a fixed time-dependent angle (e.g., RCD); (*iii*) cells moving to the right with a 90˚ turn midway through the trajectory; and (*iv*) a more realistic depiction of cell movement, including background noise, cells that enter and exit the field of view, cells that are stuck for part or the entirety of the video, and linear speed variation both between cells and within individual cell trajectories. For the final control input, referred to above as (*iv*), each cell had an inherent linear speed selected at random from a normal distribution, and each in-between frame step distance was taken from a uniform distribution spanning 0.75–1.25 times the expected step length based on that cell's inherent speed. Figure 2 depicts the final images of the video traces for each of these control experiments. Subsequent analysis of control videos (Supplementary Videos S2–S5) created in MATLAB confirmed all analytical capabilities. Supplementary Video S2 shows cells from the first control. The preprogrammed average linear speed was 15.20 μm/s, and TumbleScore returned a value of 15.31 ± 0.55 μm/s. Supplementary Video S3 shows cells moving in a straight line with a programmed average RCD of 158.73 degrees/s; TumbleScore returned an RCD of 158.65 ± 0.04 degrees/s. Supplementary Video S4 shows cells moving in identical paths, each making a 90° turn halfway through its trajectory. The preprogrammed ACR was 1.4142, and the TumbleScore value was 1.4142. Supplementary Video S5 shows cells moving with a programmed average linear speed of 12 μm/s and a 1.5 μm/s SD. TumbleScore returned a mean linear speed of 12.12 μm/s (SE = ± 0.19 μm/s) with an SD of 1.47 μm/s. It should be noted that TumbleScore function was unaffected by cells entering or exiting the frame. In addition, 20% of the cells did not move throughout the course of the video, and TumbleScore ignored all of them; 13% of cells stopped moving midway through the video, and TumbleScore ignored all of them after they ceased to move. A linear speed histogram from Supplementary Video S5 is shown in Supplementary Figure S3. While these control videos provide validation for the function of TumbleScore when confronted with common experimental issues (fluid motion, stuck cells cells moving into and out of focus, etc.), we recognize that not all issues can be addressed by hypothetical control scenarios and that there may be instances where calculated velocities are unduly biased.

Next, wild-type (WT) K-12 W3110 *E. coli* cells were tested with a *cheZ* isogenic null mutant (W3110 Δ*cheZ*), and the latter was transformed with plasmid pFZY1, which overexpresses CheZ. Average speed and ACR for these cells are indicated in Table 1. The effects of mutation and restoration were as expected, with CheZ-deficient cells having a lower average speed and a higher ACR. The cells with restored CheZ exhibited behaviors nearing those seen in WT cells. Also, the rose graphs in Figure 3 align with the speed and ACR calculations but inform more visually on directionality. Supplementary Videos S6–S8 are the motility videos that were analyzed in this study. For cells lacking CheZ (Figure 3A), there was an overall lower net displacement, or spread. A higher spread was attained by cells expressing CheZ, both WT and restored mutant. The cell trajectories appeared random, with no bias from growth conditions, genetic mutation, or background fluid flow. TumbleScore supplied conclusive evidence of an altered phenotype, both quantitatively through metrics and visually through rose graphs.

Although we developed TumbleScore for peritrichously flagellated bacteria, the program could be used to track monotrichous bacteria models (which have different motility styles) such as *Rhodobacter sphaeroides* ("run-and-stop") and *Pseudomonas aeruginosa* ("run-and-reverse"). Depending on the strain, certain metrics will be of varying relevance. In addition, although designed to accommodate videos with a slow frame rate (<10 per second), this system is also effective for videos taken with faster cameras. Users must be aware of the limits of low frame-rate technology. Previous analysis has illuminated the effect of sampling rate on the accuracy of observed metrics (26).

Here, we have demonstrated that TumbleScore can generate meaningful cell motility data in a user-friendly format from videos obtained with commonly used equipment. Refinements to the source code can be made in order to provide a more detailed analysis, if desired. TumbleScore software and complete instructions for its use are freely available at www.bentley.umd.edu.

## Author contributions

A.E.P. created the TumbleScore software, generated fake videos, and analyzed data. R.M. and W.B. had the original idea to automatically track bacteria. R.M. and C.R.V. contributed cells and filmed motility videos. H.U. assisted in software development. W.B. supervised this project. A.E.P. wrote the paper, which was edited by R.M. and W.B.

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## Competing interests

The authors declare no competing interests.

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