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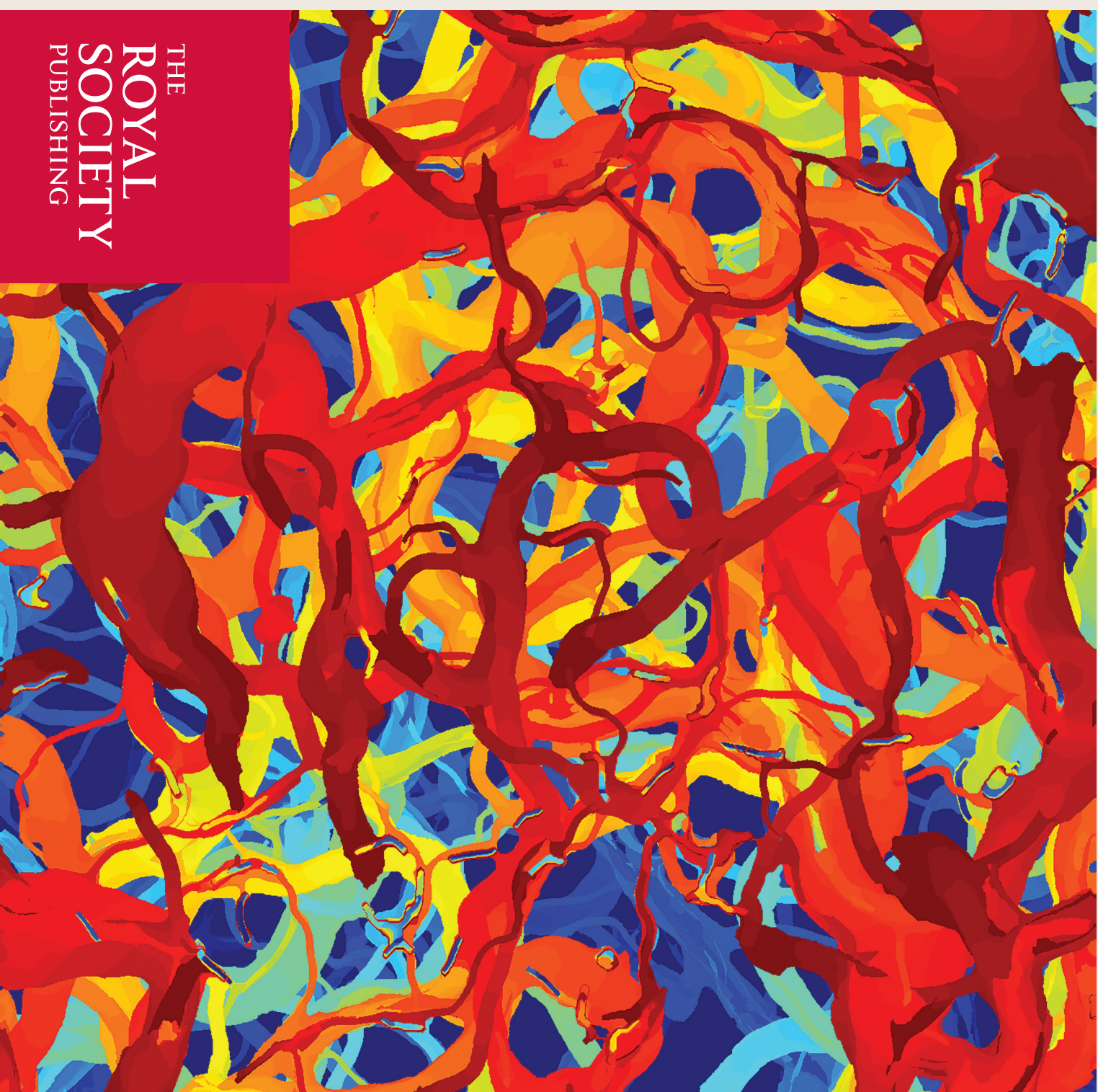
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## Research

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# Directional reversals enable *Myxococcus xanthus* cells to produce collective one-dimensional streams during fruiting-body formation

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The formation of a collectively moving group benefits individuals within a population in a variety of ways. The surface-dwelling bacterium *Myxococcus xanthus* forms dynamic collective groups both to feed on prey and to aggregate during times of starvation. The latter behaviour, termed fruiting-body formation, involves a complex, coordinated series of density changes that ultimately lead to three-dimensional aggregates comprising hundreds of thousands of cells and spores. How a loose, two-dimensional sheet of motile cells produces a fixed aggregate has remained a mystery as current models of aggregation are either inconsistent with experimental data or ultimately predict unstable structures that do not remain fixed in space. Here, we use high-resolution microscopy and computer vision software to spatio-temporally track the motion of thousands of individuals during the initial stages of fruiting-body formation. We find that cells undergo a phase transition from exploratory flocking, in which unstable cell groups move rapidly and coherently over long distances, to a reversal-mediated localization into one-dimensional growing streams that are inherently stable in space. These observations identify a new phase of active collective behaviour and answer a long-standing open question in *Myxococcus* development by describing how motile cell groups can remain statistically fixed in a spatial location.

## 1. Introduction

The collective motion of individuals that exhibit complicated group dynamics is a hallmark of living systems from single-celled bacteria to large mammals. Collective groups can gain advantages including ultra-sensitivity to perturbations, increased temporal response and increased protection from the environment [1–4]. It is often considered that individuals follow simple interaction rules that give rise to surprising group phenomena as an emergent property [5,6]. However, in many cases, how a group decides to change its behaviour, or alternatively how groups can perform multiple functions, remains unclear [7]. Do individuals have to perform increasingly more complicated tasks, or can they merely transition between a preset number of simple interaction rules to modify group behaviour [8]?

A striking manifestation of multicellular collective behaviour is the formation of dynamic cell groups by the soil-dwelling bacterium *Myxococcus xanthus* [9–11]. In a plentiful environment, the coherent motion of cells allows them to hunt prey through the cooperative production of antibiotics and digestive enzymes [12]. By contrast, if a swarm cannot find sufficient nutrients, its cells begin a complex, multi-step process that leads to the formation of giant aggregates called fruiting bodies within which many of the cells sporulate [9]. This process takes several hours and involves multiple distinct stages of

group behaviour [13]. How tens of thousands of cells can move in a coordinated fashion to form large, stationary fruiting bodies remains unsolved.

Unlike many classical aggregation phenomena where a reduction in mobility gives rise to static aggregates as the motion of the individuals becomes essentially frozen, *M. xanthus* cells and cell groups remain dynamic throughout the developmental process, often moving over long distances as fruiting bodies are born, grow, coalesce and even transiently disintegrate. These cells are able to amass cell groups that retain both cellular and group motility even as the density coarsens over time. For this reason, models that invoke a density-dependent reduction in cell speed and cell jamming fail to capture the full features of *M. xanthus* group dynamics [14]. This model necessarily yields a frustrated aggregate that cannot perform the dynamic group motions seen later in fruiting-body development. Slowing near aggregates cannot be the main driving force behind aggregation as it would merely result in a 'freezing' of the density and impair the adaptive dynamics of fruiting bodies.

A second theory in the field, although less well worked out, relates to flocking as seen in birds and fish. It is often hypothesized that *M. xanthus* cells move collectively and that this produces a type of flock that morphs into a fruiting body [8,10,15]. The problem with this view is that coherently moving and ordered groups, particularly of elongated individuals such as bacteria, are inherently unstable and cannot result in static aggregates. The instability of such aggregates is manifested in what has been termed giant number fluctuations (GNFs, where the standard deviation of the number of bacteria  $n$  grows faster than  $\sqrt{n}$ ) and spontaneous phase separation in two or more dimensions [16]. Flocking theories, therefore, suffer from two substantial problems. First, GNFs necessarily cause large inhomogeneities and the break-up of any large cell aggregates that develop. Second, flocks are obligately motile and cannot remain stationary in space. How *M. xanthus* might overcome these issues to produce large, stationary fruiting bodies remains a central mystery in the flocking theory of aggregation.

Here, we show that *M. xanthus* cells switch between two-dimensional flocks and quasi-one-dimensional streams during the initial stages of development. Flocks are used at the outset as a way of increasing group size, although groups exhibit unstable dynamics and are not fixed in space. After about an hour of the initiation of starvation and the start of the motility experiment, cells switch behaviour and form one-dimensional 'flocks' that are naturally stable and immune to the fluctuations seen in flocks in higher dimensions. One-dimensional streams are generated by a combination of the rod shape of the cells, steric effects, and reversals of cell direction and represent a new phase of active collective behaviour.

Perhaps, the most striking feature of *M. xanthus* motility is the presence of periodic directional reversals that drive unique collective modes such as wave-like dynamic ripples [17,18]. The cells, which glide in the direction of their long axis, possess a dynamic cell polarity that routinely switches direction by 180° [9]. These reversal events are accompanied by the exchange of a number of polarity and motility proteins between the leading and lagging poles which then switch roles. Reversals are critical for complex group behaviour, a link first observed by Blackhart & Zusmann [19]. Without a properly functioning reversal mechanism, *M. xanthus* cells fail to order themselves within a swarm, and non-reversing cells are impaired in their ability to

produce an expanding swarm [20]. Here, we present evidence that *M. xanthus* cells dynamically tune their reversal frequency to affect a phase transition from two-dimensional flocking to one-dimensional streaming.

A key limitation of previous studies of *M. xanthus* collective behaviour is that they were based on observing either large cell groups at low optical magnification incapable of resolving single cell behaviour, or only a small number of individuals within a large group where cell–cell dynamic interactions could not be studied. In most of these studies, only a small number of cells are analysed, either manually or semi-automatically, because of a lack of robust algorithms for tracking thousands of cells simultaneously. Recent automated approaches have been limited in scale and cannot produce long cell tracks in large dense cell clusters [21–23]. The small size of these datasets precludes an in-depth statistical study of group behaviour.

In order to develop a statistical model of *M. xanthus* group dynamics, we developed a high-throughput computational image analysis platform to measure the position and motion of each cell in a population of thousands of cells over several hours at a high temporal sampling rate (6 frames min<sup>-1</sup>; cell speed: approx. 1  $\mu\text{m min}^{-1}$ ), to bridge the dynamics of single cell motion with the emergence of mesoscale group order. Here we focus on understanding the very initial stages of fruiting-body formation, when sporadically distributed cells find each other to form clusters that merge and grow into larger cell groups. We show that reversal frequency, instead of cell speed, is the key factor that regulates the group behaviours of cells which switch from an initial flock-like searching to static aggregation. Cell–cell alignment and cellular reversals produce one-dimensional, stream-like aggregates that are inherently stable and not subject to GNFs.

## 2. Results and discussion

### 2.1. Cell tracking in densely packed groups

We imaged the motion of cells directly after starvation on an agar pad every 10 s for 4 h. To track the motion of individual cells at high local spatial densities, we developed the custom-written BCTracker bio-image informatics software (see Material and methods section) that automatically segments dense cells and tracks these cells over time. This algorithm is able to track all (approx. 1000) cells per movie, including those in large, densely packed groups. Overall, in all experiments, we detected, segmented and tracked nearly 4 million individual bacteria organized into more than 44 000 filtered tracks across three 4-h long movies for both the wild-type *M. xanthus* DZ2 strain and the reversal-deficient mutant  $\Delta\text{FrzE}$  [24], respectively. This resulted in a total of 2 159 196 and 1 763 993 tracked cells, respectively.

### 2.2. Cells aggregate into streams during changes in reversal frequency and local cell density

In our experiments, wild-type bacteria are randomly oriented and distributed at the onset of starvation as seen in figure 1a. These isolated cells find each other via a series of collisions as they glide over the substrate (figure 1b). Steric hindrance, combined with collisions and following of slime trails, lead to the local alignment of the cells whereby neighbouring cells in a group are all aligned in the same direction. These

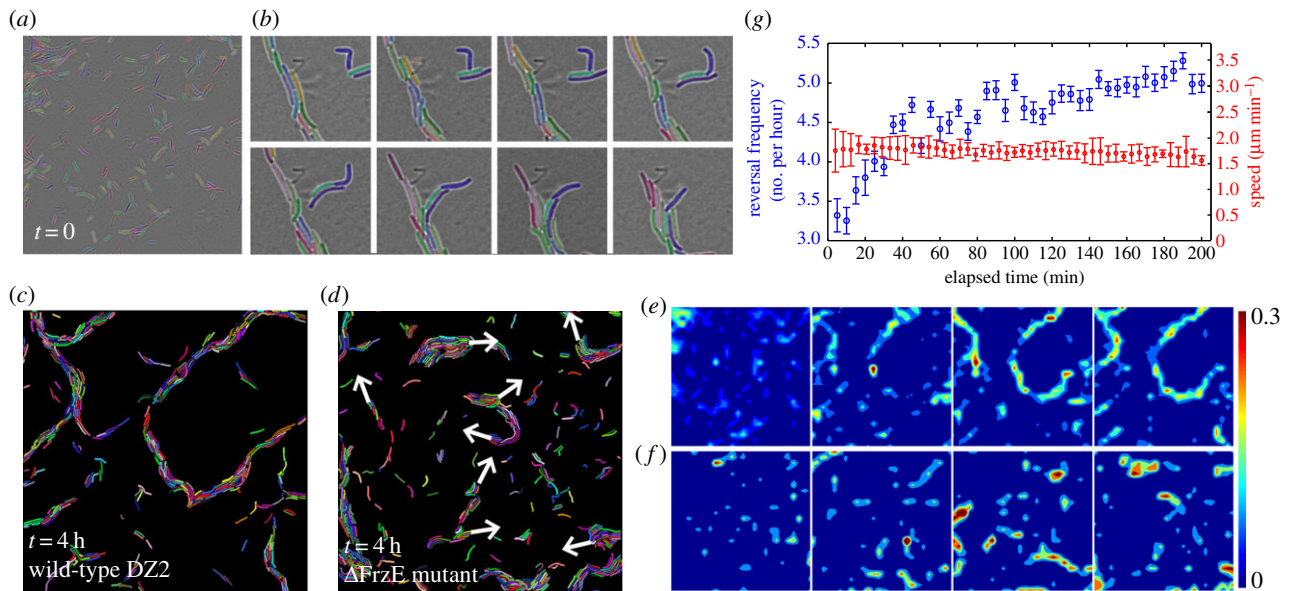


**Figure 1.** *Myxococcus xanthus* cells regulate their reversal frequencies and take advantage of increases in cell density, which are mediated by physical interactions, to form stream-like aggregates. (a) A typical dispersion of a monolayer of *M. xanthus* cells on a feature-less and nutrient-less surface of agarose gel at the start of an experiment. Individual cells are identified and marked to aid visualization. (b) Steric interactions between individual moving bacterial cells align their directions and cause linear aggregates to form over time. The sequence of images is shown every 40 s. (c) Over a duration of 4 h after starvation, the local bacterial density in the aggregates increases to approximately  $\approx 1$  cell per  $3 \mu\text{m}^2$  for the wild-type cells. The growth of the aggregates of wild-type cells is confined to preferred locations in space, which eventually form stream-like elongated aggregates. (d) Non-reversing,  $\Delta$ FrzE cells form coherently moving flocks, groups of cells that all move in the same direction (arrows). (e, f) Cell density graphs (cells  $\mu\text{m}^{-2}$ ) shown at hourly intervals for the wild-type (e) and non-reversing mutant (f). When the ability of the bacteria to reverse is suppressed, the aggregates of the non-reversing mutant are highly mobile and grow and shrink dynamically without forming streams. Scale: all individual panels are  $110 \times 110 \mu\text{m}$ . (g) The reversal frequency (blue) and speed (red) of the wild-type cells over time.

aggregates of cells, with local nematic order, move together over the course of a few hours. As the bacteria start to aggregate through these collisions, the local density of cells increases about 10-fold from approximately 0.1 cell per  $3 \mu\text{m}^2$  to approximately 1 cell per  $3 \mu\text{m}^2$  within the aggregates. For the wild-type bacteria, this increase in density is localized spatially with very dense regions surrounded by large voids (figure 1c,e; electronic supplementary material, movie S1). The combination of an elongated shape, nematic ordering and a high density, ultimately leads to the formation of stationary, stream-like aggregations of cells (figure 1e).

In order to evaluate the effect of directional reversals on this aggregation behaviour, we used a  $\Delta$ FrzE mutant strain which impairs the ability of cells to reverse. Previous studies [19,25,26] with these mutant cells have shown that they are indeed hypo-reversing, i.e. they reverse with a very long time period. However, in our experiments, we were not able to detect any reversals for the cells and refer to them as non-reversing. In these non-reversing cells, the local density of the cells increases at a similar rate to the wild-type cells (figure 1f). However, the aggregates that result are no longer elongated, stream-like or stationary. Instead, the  $\Delta$ FrzE mutants form two-dimensional flocks of collectively moving cells that appear as motile high density blobs in figure 1d,f and electronic supplementary material, movie S2. Comparing this with the wild-type cells, cells in the streams do not move cohesively in the same direction because of their periodic reversals. As a result, the streams are largely fixed in space and increase in width over time as more bacteria align and join the stream.

Over the course of the experiment, we observed a marked increase in the reversal frequency of the cells, whereas the cell speed remained constant during all 4 h of observation

(figure 1g). During the first hour after starvation, the reversal frequency of the cells rapidly increased from three reversals per hour to five reversals per hour (figure 1g). During this period, however, the speed of the bacteria remains almost constant at approximately  $1.5 \mu\text{m min}^{-1}$ . Taken together, these observations suggest that in the first hour after starvation, cells move persistently in a certain direction for a greater distance than at later times, allowing them to efficiently explore space and search for neighbours.

### 2.3. Spatio-temporal dynamics of aggregation and stream formation

The spatio-temporal dynamics of the stream formation clearly depends on the motility of the individual bacteria and its regulation, particularly of the reversal frequency of the wild-type cells, affecting the way the bacteria explore the space around them. The temporal dynamics of the exploration of space by the cells is quantified by (i) the rate at which the bacteria visit the various regions in space and (ii) the frequency with which they repeat such visits. Figure 2a shows the fraction of the total available area that is visited by the bacteria. We can characterize the temporal dynamics of this spatial exploration into two phases: an ‘exploratory phase’ and a ‘streaming phase’. In the ‘exploratory phase’, exhibited by wild-type cells during the first hour after starvation and non-reversing cells throughout the experiment, cells rapidly explore space, allowing them to search for nutrients and to initiate aggregation by finding neighbouring bacteria. An hour after starvation, wild-type cells undergo a phase transition from the flocking phase to the streaming phase. This slows down the exploration of space dramatically as the cells remain localized within the streams. The non-reversing

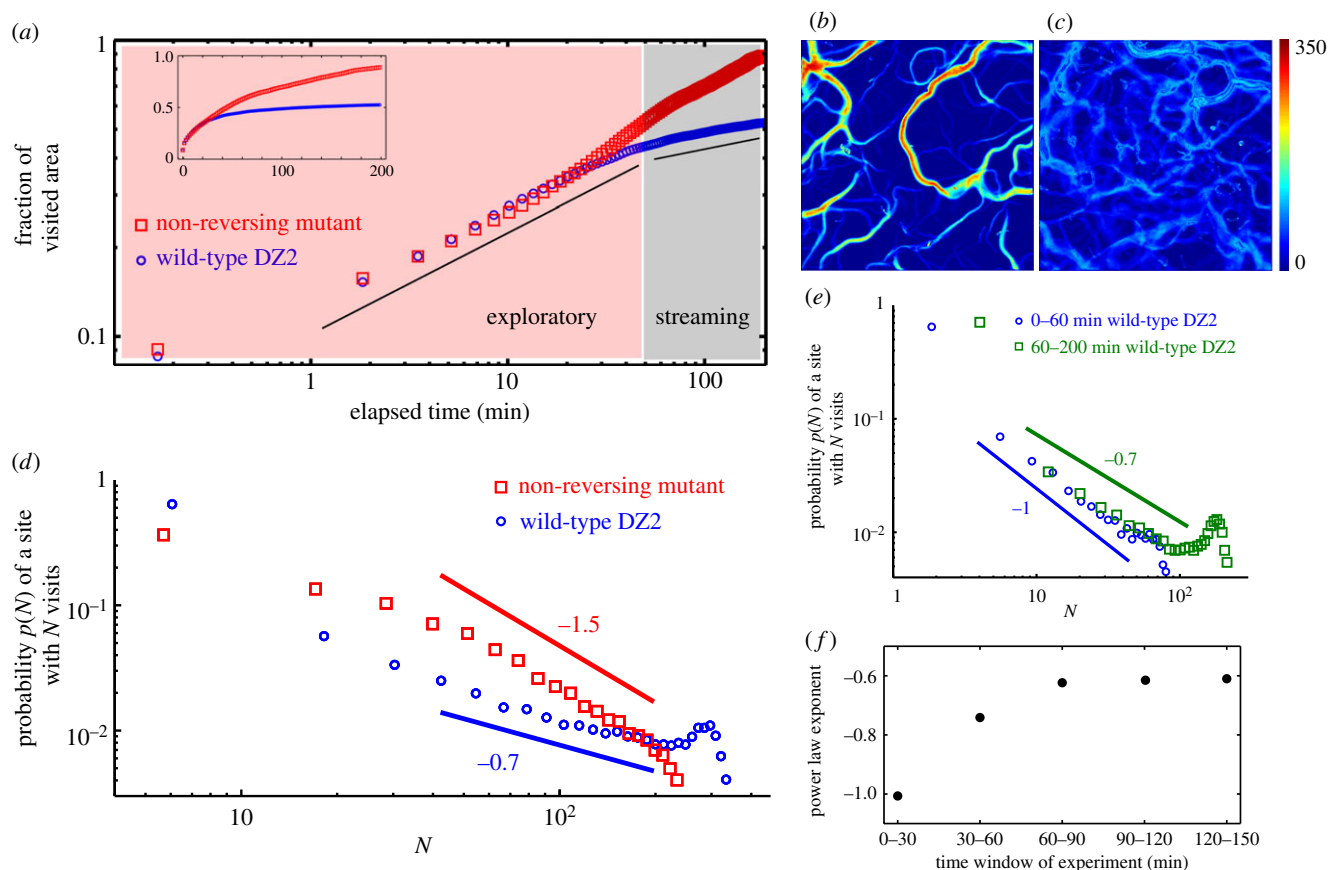


**Figure 2.** Spatial exploration by the bacteria is arrested because of the aggregation into streams and the aggregation is marked by a transition from an exploratory phase to the quasi-stationary streaming phase. (a) The dynamics of the spatial exploration by the cells. Black lines are a guide to the eye to mark the change in the slope during the ‘exploratory’ and ‘streaming’ phases. The inset shows the same data on a linear scale to show the saturation of the visited area fraction for the wild-type cells. A map of the visit frequencies for (b) the wild-type DZ2 cells during the first 4 h following starvation and (c) non-reversing  $\Delta$ FrzE cells. (d) The probability distribution of the number of visits  $N$  of a given site in space by a cell follows a power-law decay for the wild-type and  $\Delta$ FrzE mutants. The appearance of the peak in the number of visits is seen for the wild-type cells. (e) The site visit probability for the wild-type cells in the first 60 min and last 140 min of a 4 h long experiment. (f) The power-law exponent of the decay of the probability distribution (calculated from the dynamics of the cell motions in 30 min windows of the experiments).

mutants, however, continue to flock in groups over the entire 4-h experiment.

To quantify how the motion of individual cells was distributed across space, we constructed a surface-visit map for all the bacteria in a field-of-view, i.e. we probed the number of times a location in the field of view had been visited by a bacterium (see Material and methods section). We find that a considerable amount of the space remains unexplored for the wild-type bacteria (figure 2b) and the highest number of visits are localized to the region in space that eventually forms the stream. For the non-reversing  $\Delta$ FrzE mutant cells, however, the visits are distributed more evenly across space (figure 2c). To combine the information from multiple experiments, we calculated the probability distribution,  $p(N)$ , of the number of pixels with  $N$  visits from the surface-visit maps (figure 2d). Over a range of values of  $N$ , this distribution exhibits a power-law decay. While the decay exponent is approximately  $-1.5$  for the  $\Delta$ FrzE mutant, it is less steep, an exponent of  $-0.7$ , for the wild-type cells.

This power-law decay reveals features of the motility mechanism. Any feature of the system that causes bacteria to have bias for a site that they, or another cell, previously visited will affect the way space is explored when compared with an unbiased random walk. This causes some locations to be more preferable than others and may be a result of the

interaction with slime trails deposited on the substrate [27]. In addition, the steeper slope of the decay for  $\Delta$ FrzE cells indicates that they explore space more uniformly than the wild-type as is evident also from the visit map in figure 2b.

Strikingly, while  $p(N)$  decreases monotonically with  $N$  for the non-reversing bacteria, the distribution for the wild-type cells is marked by a peak in the probability at higher values of  $N$ . This peak indicates a significant probability that a few sites are preferentially visited many times. This occurs because of the formation of localized streams in which the reversing bacteria traverse over the same sites many times.

The distribution  $p(N)$  changes during the first hour of aggregation, consistent with the occurrence of a phase transition. When calculated using data from the first hour, the power-law exponent is  $-1$  and does not display the peak at large  $N$ . By contrast, the distribution from subsequent times has a slope of  $-0.7$  and contains a prominent peak at  $N \sim 200$  (figure 2e). Furthermore, the power-law exponent of the decay gradually increases over time from the flock-like value to the stream-like value (figure 2f). As time proceeds, the number of preferred locations for localization of the bacteria increases (i.e. along the stream), each drawing a large number of visits because of the reversal of the cells. The transition from the flock-like to the stream-like behaviour of the *M. xanthus* cells, which occurs after the first hour of starvation, corresponds to the fixation of

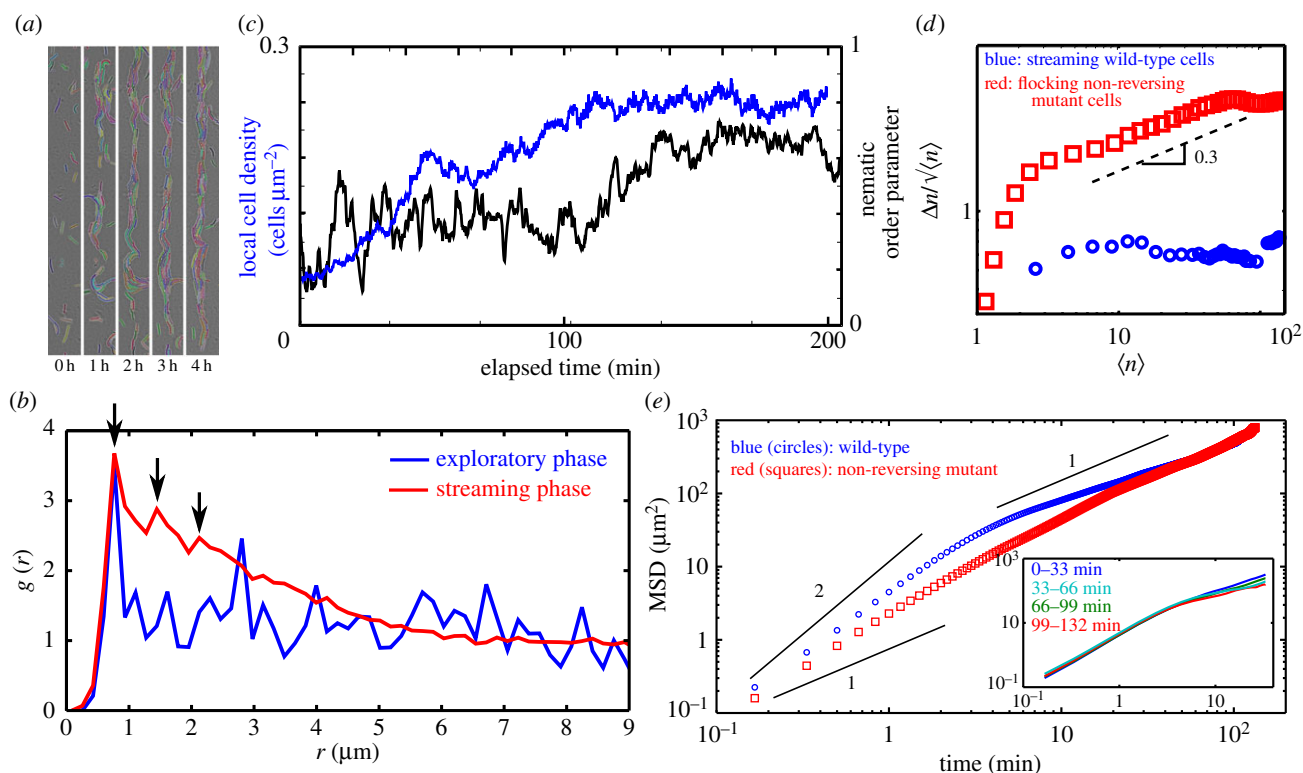


**Figure 3.** The stream-like aggregates are a quasi-one-dimensional active nematic. (a) A series of images shows the nematic like ordering of the cells along the long axis of the stream as it forms over time. (b) The radial distribution function  $g(r)$  for the bacteria in the stream region during the exploratory and the streaming phases. Data are analysed in the exploratory and streaming phases for bacteria from five different streams, each comprising a few hundred bacterial cells. (c) As the local cell density increases, the cells align along their body axis, leading to an increase in the local nematic order. The nematic order is an average over five streams, each containing a few hundred cells, from three different experiments. (d) Number fluctuations for the flocking non-reversing mutants (red) and the wild-type streams (blue). Number fluctuations are normal for the streams while they are anomalous (giant) for the flocks. (e) The mean-squared displacement (MSD) as shown for the DZ2 (blue), which makes a transition from a ballistic motion at the short timescales to an anomalous sub-diffusive behaviour at longer times. By contrast, the  $\Delta$ FrzE mutant (red) cells remain super-diffusive throughout. The MSD is an ensemble average of a few hundred cells for each of the cell types. Inset: the MSD is calculated for every 30 min time slot from the start of the experiment.

the reversal frequency after a period of increasing reversals during the exploratory phase.

## 2.4. Streams act as one-dimensional active nematic highways

Bacteria in wild-type streams are aligned much like rod-like molecules in a nematic liquid where the molecules orient themselves in a direction along their long axis [28,29]. In this ordered state, the bacteria maintain liquid-like mobility through active motion and yet remain confined. This allows them not only to navigate eventually into a fruiting body but also prevents the streams from breaking apart. Figure 3a shows the formation of a stream in a region where the bacteria are initially oriented in random directions. As these bacteria move and collide with one another, the steric interaction during the collision provides a simple physical mechanism to orient them along their body axes such that they lie parallel to each other. Once aligned in a particular direction, the bacteria then move along that direction leading to a new set of collision and realignment events with other bacteria.

The overall effect of such dynamics is the formation of a stream with all the bacteria oriented along the stream axis. During this process, the bacteria make a phase transition from an isotropic gas-like phase to a nematic-liquid-like phase in the streams. This can be seen from the radial distribution function  $g(r)$  in figure 3b. The appearance of

distinct peaks in  $g(r)$  for the streaming phase shows the liquid-like ordering of the bacteria within the stream. This liquid-like ordering also has an additional orientational ordering of the bacteria because of their elongated shape. This ordering can be quantified using an order parameter  $Q = \sqrt{\langle \cos^2 2\theta \rangle + \langle \sin^2 2\theta \rangle}$ , where  $\theta$  is the angle between the body axis of the bacteria and a direction of reference oriented along the direction of the stream. Limiting cases correspond to  $Q = 0$  for a perfectly disordered state and  $Q = 1$  when all the bacteria are perfectly aligned with their neighbours. Initially, the random orientation of the bacteria results in a low value of the order parameter  $Q \sim 0$  and as the stream builds up to reach a steady density, the ordering dynamics progressively orient the bacteria, leading to a nematic state within the stream (figure 3c).

Flocks and other active nematics in two dimensions or more are marked by the presence of GNPs, where the standard deviation  $\Delta n$  of a mean number  $n$  of active apolar particles grows faster than  $\sqrt{n}$  [16]. In general, this should lead to disruption of any aggregated groups and would be counter-productive for fruiting-body formation. These anomalous fluctuations are in contrast to more common (normal) fluctuations, where the standard deviation  $\Delta n$  grows as  $\sqrt{n}$  in accordance with the central limit theorem. For conventional systems undergoing normal fluctuations, there are no abnormally large fluctuations in the density, whereas density is not a well-defined quantity in an active nematic system.

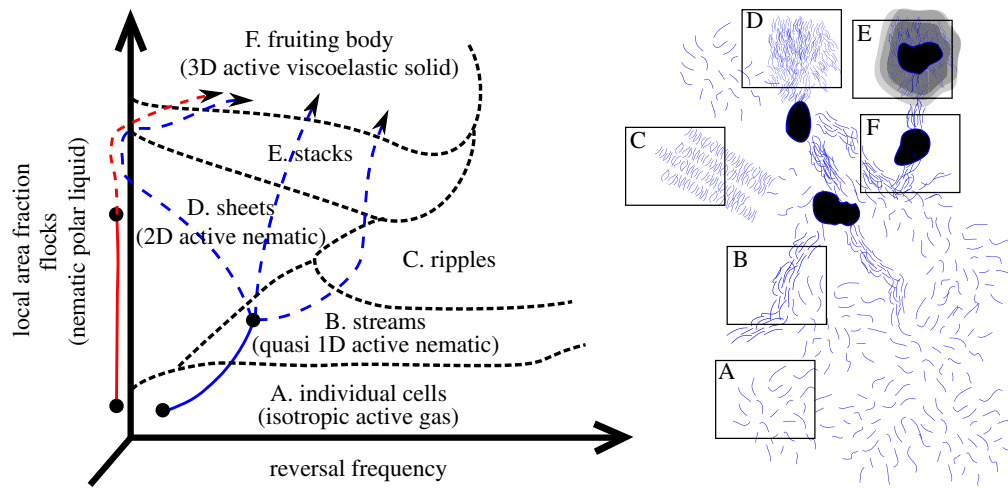


**Figure 4.** An active ordered fluids framework for the developmental cycle of *Myxococcus xanthus*. In a high-dimensional phase space, we consider local area fraction (cell density) and reversal frequency of the myxobacterial cells. The developmental programme of the bacteria is marked by various ordered states such as (A) isolated cells: an isotropic active gas phase, (B) streams: a quasi-one-dimensional active nematic fluid, (C) ripples: in which the cells are ordered in periodic rows [17] like in a smectic liquid crystalline phase [32], (D) sheets: in which the cells are ordered in a two-dimensional apolar active nematic fluid, (E) stacks: in which multiple sheets [11] are ordered in smectic-like layers on top of each other [33] and (F) fruiting bodies: an active viscoelastic solid phase [9]. The blue and red lines represent the putative paths in phase space followed by wild-type cells and non-reversing mutants, respectively. The data for the solid lines are presented in this paper.

GNFs, however, are suppressed in one dimension so that  $\Delta n \sim n^{0.5}$ . This is what we observe for bacteria in streams ([16], figure 3d blue). By contrast, the dynamic flocking behaviour of the non-reversing cells leads to anomalous giant fluctuations such that  $\Delta n \sim n^{0.5+0.3}$  as reported previously for bacteria ([10,30], figure 3d red). Therefore, even though the wild-type bacteria remain active and motile in the stream, this motility does not lead to large fluctuations in the bacterial density. This is important for cells to maintain contact with each other and ensure continuity of the aggregates.

Motion within a stream is quasi-one-dimensional, like cars along a highway. However, even though the bodies are aligned, their velocities remain uncorrelated because of reversals, i.e. the nematic alignment is apolar without a single direction of motion. This allows the stream to remain fixed in space while maintaining mobility and avoiding GNFs. A high level of mobility within the streams is revealed by examining the mean-squared displacement (MSD) as a function of time,  $t$ , for the bacteria (figure 3e). At short times, the MSD increases  $\propto t^\alpha$ , with  $\alpha \sim 2$  because of the quasi-one-dimensional directed motion of the cells. Surprisingly, at longer times, the MSD increases  $\propto t^\alpha$ , with  $\alpha < 1$  indicating a sub-diffusive, constrained motion. This is probably because of the confinement of the cell motion in the quasi-one-dimensional streams and the periodic reversals of the cell movement direction along the streams leading to head-to-head collision events [31], but further investigation is necessary. By contrast, the MSD for the non-reversing mutants remains super-diffusive (i.e.  $\text{MSD} \propto t^\alpha$ ,  $\alpha > 1$ ) for all times. Indeed, this is expected given that these cells form flocks which traverse space in a persistent random walk. The anomalously slow (sub-diffusive) growth of the MSD for the wild-type cells indicates a very slow dispersal of the cells at long times which effectively prevents the loss of localization while still keeping the cells motile.

## 2.5. An active fluids framework for *Myxococcus xanthus* development

The similarity of the collective motion of *M. xanthus* cells to the dynamics of fluids, in particular to liquid crystalline fluids,

naturally suggests an underlying self-organization principle. The various structural aggregates of the *M. xanthus* from the isolated cells to the streams and fruiting bodies is similar to the phase ordering in liquid crystalline fluids, with the switching between the different phases marked by well-defined phase transition points. The key difference between biological systems and everyday fluids is that living matter is active; the individuals within the group are self-propelled. In statistical mechanics based theories and simulations, self-propulsion together with simple physical interactions between individuals, such as collisions and steric hindrance, has been shown to lead to collective motion phases and patterns bearing a striking similarity to natural phenomena [28,29].

We propose that the developmental cycle of the *M. xanthus* can be treated as a collection of various active fluid phase behaviours (figure 4) embedded in a high-dimensional phase space involving both physical and biochemical effects. While the transitions between the phases have to be explored in future work (either via experiment or simulation), all of the different organizations of the myxo cells have already been widely reported and studied: the streams as we have shown here, the rippling phase [17], the three-dimensional stacks [33] and finally the fruiting bodies [9]. We suggest here that the transitions between the various phases can be dynamically controlled by *M. xanthus* cells via regulation of their motility and motility factors including speed, reversal frequency and potentially slime/ECM production.

Our focus in this work is a subset of this phase space involving the changes in local area fraction (areal cell density) and the reversal frequency of the *M. xanthus* cells, in which the randomly organized cells at the very onset of starvation lie close to the lower left corner and can be treated as an isotropic active gas. The fruiting bodies that result from the aggregation of the bacteria are soft mounds which are a viscoelastic solid-like phase. The route from the isotropic gas phase to this viscoelastic solid is marked by phases of different levels of ordering and relevant to the developmental cycle of the *M. xanthus*. As we have shown, the aggregation of the bacteria leads to a nematic liquid which manifests as quasi-one-dimensional streams and polar flocks. Some of these

active liquid phases and transitions between them have been shown to occur in purely physical systems such as systems of self-propelled rods [10]. However, biological activity, in addition to self-propulsion, involves chemical communication between individuals and further downstream biochemical regulation of motility and interactions between individuals. The transition between these initial stages of organization of the *M. xanthus* is presumably coordinated by the motility of the cells, their physical interactions, as well as biochemical signalling between them [34,35] and internal regulation within individuals. We have here shown, for example, that the regulation of the reversal frequencies of the cells governs one such transition. Future work defining the phases (both in two and three dimensions) and phase transitions explored by *M. xanthus* cells during predation and development, as well as the chemical and biological mechanisms that govern the control of individuals should lead to a much more in-depth understanding of how collective groups can exhibit multiple behaviours.

### 3. Material and methods

#### 3.1. Experiments and tracking

*Myxococcus xanthus* strains were grown in CYE medium (1 Casitone, 0.5 yeast extract, 10 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS), pH 7.6, 4 mM MgSO<sub>4</sub>) overnight at 32°C to OD<sub>550</sub> 0.6. For *M. xanthus* development, cells were washed in TPM (10 mM Tris-HCl, pH 7.6, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 8 mM MgSO<sub>4</sub>) three times to remove residue nutrients from CYE medium. Two microlitres of cell solution were spotted on a 1 ultra-pure agarose pad prepared on a glass cover slide (1 ultra-pure agarose dissolved in TPM medium). A glass coverslip was then covered on top of the cells.

The cells were imaged on a modified Nikon TE2000 inverted microscope with a 100× oil immersion objective (NA 1.49) using partially crossed-polarizer illumination. An image of the sample was projected onto an EMCCD camera yielding an effective pixel size of 85 nm and a total field of view of 43.5 × 43.5 μm. To increase the number of cells in our field of view, we employed a tiling strategy where a 3 × 3 grid of images was recorded every 10 s that were later post-processed into one large 110 × 110 μm image.

To remove drift during prolonged time-sequence imaging, we developed an image-based active feedback system. A z-stack of images of the central tile was taken and the sum of the Laplacian of the images was calculated as a focus index. This parameter is a strong function of the z-position and has a maximum at the highest contrast. This same auto-focus procedure was also done for every single tile at the beginning of the experiment to determine the relative best-focus position for each tile, using the central tile as the reference. Focus correction for all tiles of the image was then performed using the offsets recorded at the beginning of the experiment.

Automated tracking of individual cells and particles has been widely investigated [36–44], albeit with a focus on detecting and tracking blob-like objects such as nuclei, or point-like structures such as sub-cellular particles. Routinely used cell tracking methods do not model deformable, rod-like shapes of myxobacterial cells. A few recent studies have begun to address rod-like cells or organisms including *C. elegans* [22], or bacteria [21,23], but only for low or moderate cell densities. Tracking myxobacterial cells in our experimental set-up poses several unique challenges including: (i) non-fluorescent unlabelled cells (ii) anisotropic, rod-like shape, (iii) bending motion during gliding and

(iv) high density arising from collective cell behaviour. This combination of limiting factors is not handled by existing cell/particle tracking software, and in conjunction with the need for persistent tracks over long observation periods has motivated us to develop the BCTracker software for dense cell tracking of elongated deformable bacteria. BCTracker (electronic supplementary material, figure S1) is an automatic video analysis system for segmentation and long-term tracking of thousands of densely clustered individual bacteria, specifically designed to analyse the high spatial and temporal resolution videos collected for this study. It aims to develop an in-depth statistical study of group behaviour. The long tracks obtained from BCTracker enable characterization of reversal frequencies of cells that would not be possible with short tracklets. BCTracker expands upon our previous work in image-based cell motility analysis [37,45,46]. The key features of BCTracker that can address deformable motion and gliding behaviour across multiple scales ranging from individual bacteria, clusters, and populations are: (i) the use of a Kalman filter to model bending shape; (ii) use of spatial context through steric relationships between cells to handle high densities and (iii) combined use of active contours with explicit correspondence analysis to support accurate segmentation and tracking of deformable rod-like shapes.

The BCTracker high-throughput video analysis pipeline involves three major stages. First, the mosaicking and image enhancement module uses image-to-image registration for mosaic construction combined with several image restoration steps to compensate for illumination variation, increase contrast and filter out noise. Mosaicking is used to construct a larger field-of-view to support the accurate segmentation and tracking of larger bacteria cell groups as they undergo flocking or streaming behaviours where the groups can move between microscope fields, without sacrificing spatial resolution. Image enhancement improves subsequent detection, segmentation and tracking processes. In the second stage, the feature extraction and bacteria detection module is used to extract differential geometry and morphological image features tuned for the flexible rod-shaped cells. Analysis of the extracted feature vectors results in a multi-valued mask that identifies the foreground bacteria, background and halo regions around the cells. The multi-valued mask contains positive and negative contextual regional information and is more versatile than a typical binary cell mask. An active contour energy function uses this multi-valued mask and the fused feature set to evolve the contour adaptively to better locate, refine and segment the deformable thin rod shape of the cells more accurately. A structural analysis step uses spatio-temporal shape-based constraints like the bacteria skeleton or medial axis, and the neighbourhood relationships to model the temporal interactions between spatially adjacent cells. The results of structural analysis combined with marker propagation in time and multi-frame evidence-based correction is essential for identifying and correctly segmenting touching cells. This is critical to handle the high density of clustered cells and their curvilinear shapes. The third stage is for building long persistent tracks using data association and track generation based on correspondence graphs [47]. The module involves temporal correspondence analysis, track operations such as initialization, extension, termination, recovery and linking. Building long trajectories requires reasoning about entering and exiting cells, and track splits and merges to recover from various types of segmentation errors and accurately handle the gliding or streaming motion of cells.

In order to evaluate the performance of BCTracker, we manually segmented all the cells in one field, by manually tracking the medial axes of individual cells, over a total of 100 frames. Mosaiced images used in the study consist of nine fields co-registered together to construct a single larger field-of-view. We used 50 consecutive frames from the wild-type *M. xanthus* DZ2 strain and 50 consecutive frames from the reversal-deficient mutant FrzE to generate the manual ground truth. We have compared

the automatically detected and segmented cells with the manual ground truth using our automatic correspondence analysis algorithm [48]. Overall, 8518 cells were segmented manually. For the same set of frames, BCTracker detected 8007 cells. The BCTracker cell detection recall and precision percentages are 91.43% and 97.26%, respectively, including partial matches or overlaps. Using a stricter criterion of only one-to-one perfect matches between ground-truth cells and detected cells, discarding any partial match caused by fragmented or merged cells, results in recall and precision values of 79.56% and 84.64%, respectively.

### 3.2. Data analysis

We analysed six, 4-h-long movies using BCTracker yielding nearly 4 million bacterial instances. To generate the surface-visit maps, the number of visits made by a bacterium was counted for each pixel in the field of view [49]. A histogram of these pixel visits then comprises the visit probability  $p(N)$ .

Cell reversals are marked by detecting a change in the sign of the difference of the cell velocities in two successive time points. The total number of reversals in the entire ensemble are then calculated for a given time point. Finally, the reversal frequency is calculated from the ensemble average of these reversal events over a 5 min time window.

Number fluctuations were derived from time-series data of the number of bacteria in subsystems of various sizes (from  $4 \times 4$  to  $110 \times 100 \mu\text{m}$ ). From each time series, the mean

number of bacteria  $n$  and the standard deviation  $\Delta n$  was measured. The magnitude of the number fluctuations was then quantified by the deviation in the mean number  $\delta N$  and normalized by  $\sqrt{n}$ . For the non-reversing mutant, data from three movies (each 4 h long) were used, whereas data from five elongated streams were used for the wild-type cells.

The radial distribution function  $g(r)$ , was calculated from the positions  $r_i$  of the bacteria using

$$g(r) = \frac{1}{\rho} \left\langle \sum_{i \neq 0} \delta(r - r_i) \right\rangle,$$

where  $\langle \dots \rangle$  denotes the ensemble average.

**Authors' contribution.** S.T., M.S. and J.W.S. conceived and designed the research; M.S. performed experiments; M.S., F.B. and K.P. developed the BCTracker and related analysis; S.T. performed analysis; and S.T. and J.W.S. wrote the manuscript.

**Competing interests.** We declare we have no competing interests.

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## References

- Cavagna A, Cimarelli A, Giardina I, Parisi G, Santagati R, Stefanini F, Viale M. 2010 Scale-free correlations in starling flocks. *Proc. Natl Acad. Sci. USA* **107**, 11 865–11 870. (doi:10.1073/pnas.1005766107)
- Zitterbart DP, Wienecke B, Butler JP, Fabry B. 2011 Coordinated movements prevent jamming in an emperor penguin huddle. *PLoS ONE* **6**, 5–7. (doi:10.1371/journal.pone.0020260)
- Ancel A, Visser H, Handrich Y, Masman D, Le Maho Y. 1997 Energy saving in huddling penguins. *Nature*, **385**, 304–305. (doi:10.1038/385304a0)
- Berdahl A, Torney CJ, Ioannou CC, Faria JJ, Couzin ID. 2013 Emergent sensing of complex environments by mobile animal groups. *Science* **339**, 574–576. (doi:10.1126/science.1225883)
- Moussad M, Helbing D, Theraulaz G. 2011 How simple rules determine pedestrian behavior and crowd disasters. *Proc. Natl Acad. Sci. USA* **108**, 6884–6888. (doi:10.1073/pnas.1016507108)
- Herman R, Prigogine I. 1979 A two-fluid approach to town traffic. *Science* **204**, 148–151. (doi:10.1126/science.204.4389.148)
- Parrish JK, Edelstein-Keshet L. 1999 Complexity, pattern, and evolutionary trade-offs in animal aggregation. *Science* **284**, 99–101. (doi:10.1126/science.284.5411.99)
- Zhang Y, Ducret A, Shaevitz J, Mignot T. 2012 From individual cell motility to collective behaviors: insights from a prokaryote, *Myxococcus xanthus*. *FEMS Microbiol. Rev.* **36**, 149–164. (doi:10.1111/j.1574-6976.2011.00307.x)
- Zusman DR, Scott AE, Yang Z, Kirby JR. 2007 Chemosensory pathways, motility and development in *Myxococcus xanthus*. *Nat. Rev. Microbiol.* **5**, 862–872. (doi:10.1038/nrmicro1770)
- Peruani F, Starruss J, Jakovljevic V, Sogaard-Andersen L, Deutsch A, Bar M. 2012 Collective motion and nonequilibrium cluster formation in colonies of gliding bacteria. *Phys. Rev. Lett.* **108**, 1–5. (doi:10.1103/PhysRevLett.108.098102)
- Starruss J, Peruani F, Jakovljevic V, Sogaard-Andersen L, Deutsch A, Bar M. 2012 Pattern-formation mechanisms in motility mutants of *Myxococcus xanthus*. *Interface Focus* **2**, 774–785. (doi:10.1098/rsfs.2012.0034)
- Rosenberg E, Keller KH, Dworkin M. 1977 Cell density-dependent growth of *Myxococcus xanthus* on casein. *J. Bacteriol.* **129**, 770–777.
- Shimkets LJ. 1990 Social and developmental biology of the myxobacteria. *Microbiol. Rev.* **54**, 473–501.
- Slusarenko O, Zusman DR, Oster G. 2007 Aggregation during fruiting body formation in *Myxococcus xanthus* is driven by reducing cell movement. *J. Bacteriol.* **189**, 611–619. (doi:10.1128/JB.01206-06)
- Starruss J, Bley T, Sogaard-Andersen L, Deutsch A. 2007 A new mechanism for collective migration in *M. xanthus*. *J. Stat. Phys.* **128**, 269–286. (doi:10.1007/s10955-007-9298-9)
- Aditi Simha R, Ramaswamy S. 2002 Statistical hydrodynamics of ordered suspensions of self-propelled particles: waves, giant number fluctuations and instabilities. *Phys. A* **306**, 262–269. (doi:10.1016/S0378-4371(02)00503-4)
- Shimkets LJ, Kaiser D. 1982 Induction of coordinated movement of *Myxococcus xanthus* cells. *J. Bacteriol.* **152**, 451–461.
- Mauriello EMF, Mignot T, Yang Z, Zusman DR. 2010 Gliding motility revisited: how do the myxobacteria move without flagella? *Microbiol. Mol. Biol. Rev.* **74**, 229–249. (doi:10.1128/MMBR.00043-09)
- Blackhart BD, Zusman DR. 1985 'Fizzy' genes of *Myxococcus xanthus* are involved in control of frequency of reversal of gliding motility. *Proc. Natl Acad. Sci. USA* **82**, 8767–8770. (doi:10.1073/pnas.82.24.8767)
- Wu Y, Kaiser AD, Jiang Y, Alber MS. 2009 Periodic reversal of direction allows myxobacteria to swarm. *Proc. Natl Acad. Sci. USA* **106**, 1222–1227. (doi:10.1073/pnas.0811662106)
- Chen J, Harvey CW, Alber MS, Ziyi Chen D. 2014 A matching model based on earth mover's distance for tracking *Myxococcus xanthus*. *Lect. Notes Comput. Sci.* **8674**, 113–120. (doi:10.1007/978-3-319-10470-6\_15)
- Roussel N, Morton CA, Finger FP, Roysam B. 2007 A computational model for *C. elegans* locomotory behavior: application to multiworm tracking. *IEEE Trans. Biomed. Eng.* **54**, 1786–1797. (doi:10.1109/TBME.2007.894981)
- Slusarenko O, Heinritz J, Emonet T, Jacobs-Wagner C. 2011 High-throughput, subpixel precision analysis of bacterial morphogenesis and intracellular spatio-temporal dynamics. *Mol. Microbiol.* **80**, 612–627. (doi:10.1111/j.1365-2958.2011.07579.x)
- Li Y, Bustamante VH, Lux R, Zusman D, Shi W. 2005 Divergent regulatory pathways control A and S motility in *Myxococcus xanthus* through FrzE,

- a CheA-CheY fusion protein. *J. Bacteriol.* **187**, 1716–1723. (doi:10.1128/JB.187.5.1716-1723.2005)
25. Kearns DB, Shimkets LJ. 2004 Chemotaxis in a gliding bacterium. *Proc. Natl. Acad. Sci. USA* **95**, 11 957–11 962. (doi:10.1073/pnas.95.20.11957)
  26. Bustamante VH, Martínez-Flores I, Vlamakis HC, Zusman DR. 2004 Analysis of the Frz signal transduction system of *Myxococcus xanthus* shows the importance of the conserved C-terminal region of the cytoplasmic chemoreceptor FrzCD in sensing signals. *Mol. Microbiol.* **53**, 1501–1513. (doi:10.1111/j.1365-2958.2004.04221.x)
  27. Burchard RP. 1982 Trail following by gliding bacteria. *J. Bacteriol.* **152**, 495–501.
  28. Marchetti MC, Joanny JF, Ramaswamy S, Liverpool TB, Prost J, Rao M, Aditi Simha R. 2013 Hydrodynamics of soft active matter. *Rev. Mod. Phys.* **85**, 1143–1189. (doi:10.1103/RevModPhys.85.1143)
  29. Ramaswamy S. 2010 The mechanics and statistics of active matter. *Annu. Rev. Condens. Matter Phys.* **1**, 323–345. (doi:10.1146/annurev-conmatphys-070909-104101)
  30. Zhang HP, Be'er A, Florin E-L, Swinney HL. 2010 Collective motion and density fluctuations in bacterial colonies. *Proc. Natl. Acad. Sci. USA* **107**, 13 626–13 630. (doi:10.1073/pnas.1001651107)
  31. Richards PM. 1977 Theory of one-dimensional hopping conductivity and diffusion. *Phys. Rev. B* **16**, 1393–1409. (doi:10.1103/PhysRevB.16.1393)
  32. Adhyapak TC, Ramaswamy S, Toner J. 2013 Live soap: stability, order, and fluctuations in apolar active smectics. *Phys. Rev. Lett.* **110**, 1–5. (doi:10.1103/PhysRevLett.110.118102)
  33. Kaiser D, Warrick H. 2014 Transmission of a signal that synchronizes cell movements in swarms of *Myxococcus xanthus*. *Proc. Natl. Acad. Sci. USA* **111**, 13 105–13 110. (doi:10.1073/pnas.1411925111)
  34. Pathak DT, Wei X, Bucuvalas A, Haft DH, Gerloff DL, Wall D. 2012 Cell contact-dependent outer membrane exchange in myxobacteria: genetic determinants and mechanism. *PLoS Biol.* **8**, e1002626. (doi:10.1371/journal.pcbi.1002626)
  35. Kim SK, Kaiser D. 1990 C-factor: a cell-cell signaling protein required for fruiting body morphogenesis of *M. xanthus*. *Cell* **61**, 19–26. (doi:10.1016/0092-8674(90)90211-V)
  36. Mukherjee DP, Ray N, Acton ST. 2004 Level set analysis for leukocyte detection and tracking. *IEEE Trans. Image Process.* **13**, 562–572. (doi:10.1109/TIP.2003.819858)
  37. Bunyak F, Palaniappan K, Nath SK, Baskin TI, Dong G. 2006 Quantitative cell motility for *in vitro* wound healing using level set-based active contour tracking. In *Proc. 3rd IEEE Int. Symp. Biomed. Imaging (ISBI)*, Arlington, VA, 6–9 April, pp. 1040–1043. Piscataway, NJ: IEEE.
  38. Mosig A, Jäger S, Wang C, Nath S, Ersoy I, Palaniappan K, Chen S-S. 2009 Tracking cells in life cell imaging videos using topological alignments. *Algorithms Mol. Biol.* **4**, 10. (doi:10.1186/1748-7188-4-10)
  39. Chen Y, Ladi E, Herzmark P, Robey E, Roysam B. 2009 Automated 5-D analysis of cell migration and interaction in the thymic cortex from time-lapse sequences of 3-D multi-channel multi-photon images. *J. Immunol. Methods* **340**, 65–80. (doi:10.1016/j.jim.2008.09.024)
  40. Kanade T, Yin Z, Bise R, Huh S, Eom S, Sandbothe MF, Chen M. 2011 Cell image analysis: algorithms, system and applications. In *IEEE Workshop on Applications of Computer Vision (WACV)*, pp. 374–381. Piscataway, NJ: IEEE.
  41. Ng MR, Besser A, Danuser G, Brugge JS. 2012 Substrate stiffness regulates cadherin-dependent collective migration through myosin-ii contractility. *J. Cell Biol.* **199**, 545–563. (doi:10.1083/jcb.201207148)
  42. Meijering E, Dzyubachyk O, Smal I. 2012 Methods for cell and particle tracking. *Methods Enzymol.* **504**, 183–200. (doi:10.1016/B978-0-12-391857-4.00009-4)
  43. Chenouard N *et al.* 2014 Objective comparison of particle tracking methods. *Nat. Methods* **11**, 281–289. (doi:10.1038/nmeth.2808)
  44. Solorzano CO, Munoz-Barrutia A, Meijering E, Kozubek M. 2015 Towards a morpho-dynamic model of the cell. *IEEE Signal Processing Magazine*, January 2015.
  45. Palaniappan K, Bunyak F, Nath S, Goffeney J. 2009 Parallel processing strategies for cell motility and shape analysis. In *High-throughput image reconstruction and analysis*, 39–87. Norwood, MA: Artech House, Inc.
  46. Ersoy I, Bunyak F, Higgins J, Palaniappan K. 2012 Coupled edge profile geodesic active contours for red blood cell flow analysis. In *9th IEEE Int. Symposium Biomedical Imaging*, 2–5 May, Barcelona, Spain, pp. 748–751. Piscataway, NJ: IEEE. (doi:10.1109/ISBI.2012.6235656)
  47. Nath SK, Bunyak F, Palaniappan K. 2006 Robust tracking of migrating cells using four-color level set segmentation. *Lect. Notes Comput. Sci.* **4179**, 920–932. (doi:10.1007/11864349\_84)
  48. Hafiane A, Bunyak F, Palaniappan K. 2008 Fuzzy clustering and active contours for histopathology image segmentation and nuclei detection. *Lect. Notes Comput. Sci.* **497**, 903–914. (doi:10.1007/978-3-540-88458-3\_82)
  49. Zhao K, Tseng BS, Beckerman B, Jin F, Gibiansky ML, Harrison JJ, Luijten E, Parsek MR, Wong GCL. 2013 Psl trails guide exploration and microcolony formation in *Pseudomonas aeruginosa* biofilms. *Nature* **497**, 388–391. (doi:10.1038/nature12155)