Self-propelled colloids constitute an important class of intrinsically non-equilibrium matter. Typically, such a particle moves ballistically at short times, but eventually changes its orientation, and displays random-walk behaviour in the long-time limit. Theory predicts that if the velocity of non-interacting swimmers varies spatially in 1D, $v(x)$, then their density $\rho(x)$ satisfies $\rho(x) = \rho(0)v(0)/v(x)$, where $x = 0$ is an arbitrary reference point. Such a dependence of steady-state $\rho(x)$ on the particle dynamics, which was the qualitative basis of recent work demonstrating how to ‘paint’ with bacteria, is forbidden in thermal equilibrium. Here we verify this prediction quantitatively by constructing bacteria that swim with an intensity-dependent speed when illuminated and implementing spatially-resolved differential dynamic microscopy (sDDM) for quantitative analysis over millimeter length scales. Applying a spatial light pattern therefore creates a speed profile, along which we find that, indeed, $\rho(x)v(x) =$ constant, provided that steady state is reached.
Escherichia coli so that its applicability to real systems is open to doubt. Theoretical derivations do not include hydrodynamic interactions, templated active self assembly10. Our strain templated self assembly using light-activated motile bacteria10,11.

Schnitzer8. Later, Tailleur and Cates showed that it is valid for dissipating states of matter, which include all living organisms, are intrinsically non-equilibrium, and give rise to new physics. Tailleur and Cates5,6 predicted that the resulting density distribution should be dissipative states of matter, which include all living organisms. Such RTPs, whose run speed can be expressed as Ran, losing directional memory: wild-type (WT) cells are RTPs15. We use sDDM to confirm that indeed \(\rho(x)v(x) = \text{constant},\) provided that steady state is reached.

### Results

**Light controlled *E. coli* and spatially resolved analysis.** Each *E. coli* is an \(\approx 2 \times 1 \mu m\) spherocylinder with \(7\text{–}10 \mu m\) helical flagella powered by rotary motors14. When all flagella rotate counterclockwise (seen from behind), they bundle and propel the cell. Every \(v_{\text{run}} \sim 1 \text{ s}^-1\) or so, one or more flagella reverse and unbundle, causing a change in direction: wild-type (WT) cells are RTPs15. At a typical average speed \(\bar{v} \geq 10 \mu m \text{ s}^-1\), they random walk with a persistence length \(L_p \sim v_{\text{run}} \sim 10 \mu m\). Deleting the cheY gene prevents tumbling; cells become ABPs with \(D_{\text{rot}}^{-1} \sim 10 \text{ s}\) and \(L_p \sim v_{\text{run}}D_{\text{rot}}^{-1} \sim 100 \mu m\), so that at times \(\gg D_{\text{rot}}^{-1}\) cells random walk with \(D_{\text{eff}} \sim v_{\text{run}}D_{\text{rot}}^{-1} \sim 10^3 \mu m^2 \text{ s}^-1\).

Our *E. coli* mutants carried a plasmid expressing proteorhodopsin (PR), which pumps protons when exposed to green light17. Cells suspended in nutrient-free motility buffer were sealed into 20 \mu m high compartments and imaged using 10X phase contrast microscopy. After some minutes, \(v\) dropped abruptly to zero upon oxygen exhaustion10. Thereafter, cells only swim when illuminated in green10,18,19, with an average speed \(\bar{v}\) that increased with the light intensity, \(I\), saturating at \(v_{\text{max}}\) beyond some \(I_0\). These are living analogues of synthetic light-activated colloids20,21.

We first used a digital mirror device10 to project a binary (bright-dark) spatial intensity pattern \(I(x,y)\) spelling out ‘UoE’ (inset of Fig. 1a) onto a field of cells that had been uniformly illuminated for some time, so \(v\) was initially constant in space. We used sDDM to measure \(v(x,y), \beta(x,y)\) and \(\rho(x,y)\) in \(64 \times 64\) (pixel\(^2\)) tiles (see Methods section and Supplementary Note 1 for details). The projected \(I(x,y)\) was rapidly replicated in a pattern of \(v(x,y)\) (Fig. 1a). A similar \(\rho(x,y)\) pattern soon forms (Fig. 1b) so that this effect can be used for templated self assembly10,11. Given that higher cell densities occur in darker regions with lower swimming speed, Eq. (3) is clearly qualitatively correct10,11. We now proceed to test it quantitatively.

### Quantitative Analysis

#### Demonstration of spatially-resolved dynamic differential microscopy.

Projected light patterns onto a sample of light-powered *E. coli* AD10 (at OD = 1) leads to a spatial variation of the mean swimming speeds \(\bar{v}\). A Map of \(\bar{v}\) as measured by sDDM when the negative ‘UoE’ pattern shown in inset is projected onto the sample. B Phase contrast image about 20 s after applying the pattern, showing accumulation in the darker regions. Magenta lines indicate the square tiles of 64 pixels (90 \mu m) size used for the sDDM analysis.
We projected a quasi-1D stepped illumination pattern

$$I(x, y) = \begin{cases} I_y, & x < 0 \\ I_y - I_y^+, & x > 0 \end{cases}$$  \hspace{1cm} (4)

on a field of these cells. This generated a spatial pattern of swimming speed, $\bar{v}(x, y)$, and cell density, $\rho(x, y)$, which we quantified using sDDM. Averaging over $y$ gives $\bar{v}(x)$ and $\rho(x)$, which allows a direct test of Eq. (3), provided that this light pattern (Eq. (4)) generates a corresponding sharp pattern of cell run speeds:

$$\bar{v}(x) = \begin{cases} \bar{v}_+, & x < 0 \\ \bar{v}_- < \bar{v}_+, & x > 0 \end{cases}$$  \hspace{1cm} (5)

This requires cells that respond rapidly to changes in $I_y$, which was found not to be the case for previous PR-expressing mutants with otherwise intact metabolism. Indeed, a recent attempt to verify Eq. (3) using PR-bearing *E. coli* found instead (in our notation) $\rho = (a/\bar{v}) + b$ with positive constants $a$ and $b$. The latter was ascribed to a long $\tau_{\text{stop}}$, which led to memory effects.

We achieved rapid response by deleting the unc gene cluster encoding the F$_{1}$F$_{-}$-ATPase membrane protein complex from a parent K12-derived ΔcheY mutant, giving a fast-responding smooth-swimmer, AD10. In fully-oxygenated phosphotolitity buffer (MB) at optical density $OD = 1$, $\bar{v} \approx 30 \mu m s^{-1}$ and a fraction $\beta \lesssim 20\%$ of cells were non-motile. When illuminated anerobically, $\bar{v}_{\text{max}} = 28 \mu m s^{-1}$ and $\tau_{\text{stop}} \ll 1 s$, compared with a $\tau_{\text{stop}}$ of many minutes in the parent strain without unc deletion. (Details of other strains we used are given in the methods section.)

Strictly speaking, a non-interacting limit does not exist for bacterial suspensions. Cells interact hydrodynamically at any concentration, although simulations show that swimmers behave effectively as non-interacting when $\rho \rho_0 \lesssim 0.1$, where $\rho_0$ is the density for the onset of collective behaviour. We observed collective motion in our *E. coli* suspension at $OD \geq 10$, corresponding to a cell body volume fraction of $\phi \approx 1.4\%$, consistent with a previous estimate of 2%.22,23, so that a quasi-non-interacting limit is reached at $OD \leq 1$. It was not possible to work below this limit because of an increasing fraction of cells trapped in circular trajectories (due to hydrodynamic interactions with walls of the sample chamber) that did not explore the whole sample compartment, hindering relaxation towards a steady state. We therefore worked at $OD \geq 1$. We report first data for $OD = 5$ ($\rho = 5 \times 10^9$ cells/ml; $\phi \approx 0.7\%$) before discussing OD = 1, where the data are noisier due to lower numbers.

**Stepped light pattern at OD = 5.** A field of AD10 cells rendered stationary by oxygen exhaustion was uniformly illuminated for $\approx 20$ min to achieve saturation speed. The light was then attenuated to $I_y$, the level of the darker half of the target pattern (Eq. (4)) for 5 min to determine $\bar{v}_- = 6.5(2) \mu m s^{-1}$. Returning the intensity to its initial level, we waited another 5 min for the swimming speed to return to $\bar{v}_+ = 13.2(2) \mu m s^{-1}$. We measured the cell density $\rho_0$ and non-motile fraction $\beta_0$ of this high-speed uniform sample, and then switched on a stepped pattern (Eq. (4)) by reducing the intensity in the $x > 0$ half plane.

Figure 2a shows the mean swimming speed averaged over $y$ tiles, $\langle \bar{v}(x,y) \rangle_y = \bar{v}(x)$, normalised to the whole-sample-averaged speed, $\langle \bar{v} \rangle$, plotted against $x$ at 30 min after switching on the stepped pattern. A stepped speed pattern was developed (Fig. 2a, black circles).

If Eq. (3) is valid, we expect the swimmer density to obey

$$\rho^s(x)\bar{v}_+(x) = \rho^s(x)\bar{v}_-(x),$$  \hspace{1cm} (6)

where ‘±’ subscripts having their obvious meanings. If the density of non-motile cells is constant throughout the experiment (see Supplementary Note 2 for justification), i.e.

$$\rho^s_{\pm} = \beta_0 \rho_0,$$  \hspace{1cm} (7)

we can write the total cell density on the two sides of $x = 0$ as

$$\rho_\pm = \rho^s_\pm + \rho^m = \rho^s_\pm + \beta_0 \rho_0.$$  \hspace{1cm} (8)

Finally, the average cell density is

$$\rho_0 = \frac{1}{2} (\rho_+ + \rho_-).$$  \hspace{1cm} (9)

Equations (6)–(9) together predict the density of motile cells in the two half-planes:

$$\rho^s_\pm = 2 \rho_0 (1 - \beta_0) \frac{1 + \beta_0}{v_\pm}.$$  \hspace{1cm} (10)

We calculated the swimmer density in our experiments from the measured total cell density $\rho(x)$ and non-motile fraction $\beta(x)$ using $\rho^s(x) = \rho(x) (1 - \beta(x))$, and normalised it by the whole-sample-averaged swimmer density. This function 30 min after the imposition of the stepped intensity pattern is also stepped (Fig. 2a, blue squares), with the theoretical predictions from Eq. (10), using the measured average $v_\pm$ as inputs (Fig. 2a, dotted line), giving a reasonable account of the step amplitude. A more sophisticated version of this model which takes the measured shape of $\bar{v}(x)$ into account (see Supplementary Note 2 for details)
is able to capture the amplitude of the jump in $\rho v(x)$ at $x = 0$ even more precisely (Fig. 2a, dashed line).

The product $\rho v(x)$ normalised to the whole sample average (Fig. 2a, red $x$’s), is indeed constant for $|x| \lesssim 200 \mu$m, verifying Eq. (6), which is the application of Eq. (3) to our conditions. However, there are systematic deviations from constancy at $|x| \gtrsim 200 \mu$m. One possible explanation is the emergence of collective motion with associated local nematic ordering\(^{23}\), which would invalidate the derivation of Eq. (3). However, we only observed collective motion at OD $\gg 10$. Instead, the deviation of $\rho v$ from constancy at $|x| \gtrsim 200 \mu$m is a kinetic effect. Figure 2b shows the time evolution of the normalised $\rho v(x)$ at different $x$. Steady state was reached rapidly for $|x| \lesssim 200 \mu$m, but was not reached by $30$ min at the extremes of our observation window, $|x| \gtrsim 600 \mu$m. Given their effective diffusivity $D_{\text{eff}} \sim 10^9 \mu$m$^2$s$^{-1}$, cells at the extremites of our compartment take $\gg 30$ min to sufficiently sample both speed regions, preventing the attainment of steady state within our observational time window. This leads to the deviations between observed and predicted $\rho v(x)$ away from $x = 0$. Nevertheless, Fig. 2b suggests that $\rho v$ = constant should be attained at all $x$ in the long-time limit.

**Stepped pattern at other cell densities.** Measurements and model predictions for the lower OD = 1 are shown in Fig. 3. The data are noisier, but show the same trends. In the vicinity of $x = 0$, $\rho v \approx$ constant. To highlight the behaviour in the two 90 $\mu$m-wide stripes of tiles bordering $x = 0$, we plot $\rho v(x)$ at $t = 30$ min for these two stripes against each other for a number of independent experiments (Fig. 4, black solid circles). In all cases, $\rho v(x) = \text{constant}$ for these central stripes to within experimental uncertainties. The ratio of $\rho v(x)$ in these two stripes plotted against the ratio of the swimming speed on the two half-planes (Fig. 4 inset, black solid circles) is consistent with this claim.

We performed experiments using the stepped light pattern at other cell densities and also using an additional smooth swimming strain (DM1). In all cases up to OD = 8, we find that $\rho v(x)$ is constant across the two central stripe of tiles on either side of $x = 0$ (Fig. 4), where we are certain that a steady state has been reached, verifying Eq. (3) up to $\rho \approx 8 \times 10^9$ cells/ml. Spatial maps and time evolution of spatial profiles for our highest density sample (OD = 8) are shown in Supplementary Figs. 6 and 7, respectively.

**Measurements using a periodic light pattern.** The complicating factor so far is the slow global convergence towards $\rho v(x)/v(x) = \text{const}$, so that steady state will only be reached in approximately 4$-$5 hours. Experiments on such time scales are impractical due to mechanical and biological stability issues. Thus, we only have direct evidence for the validity of Eq. (3) in the vicinity of the intensity step at $x = 0$. This suggests that the use of a series of thin stripes would give more clear-cut results unencumbered by kinetic issues. We found that this was indeed the case.

In response to the imposition of a one-dimensional square-wave illumination pattern of brighter-darker stripes with 540 $\mu$m repeat generated by a digital mirror device\(^{10}\), the swimming speed of bacteria changed from a uniform distribution to a square-wave distribution almost instantaneously (in $\lesssim 1$ s) (Fig. 5a). This in turn modified the cell density (initially uniform at OD = 1), which approached a steady state much more quickly. This is possible not only because of the length scale reduction, but also because swimmers can enter (say) a high-intensity region from the other cell densities and also using an additional smooth swimming speed.

Figure 5b shows the normalised swimmer density $\rho v(x)/\langle \rho v \rangle$ after 15 min of patterned illumination, together with $v(x)/\langle v \rangle$ and their product. While the data are again somewhat noisy because

**Fig. 3** Response of AD10 at OD = 1 to a stepped intensity pattern. The same quantities as in Fig. 2 are shown. Qualitatively the behaviour is the same as for the higher density, but the data are noisier due to the overall weaker signal. See Supplementary Figs. 4 and 5 for spatial maps and time evolutions, respectively.

**Fig. 4** $\rho v$ either side of an intensity step for various sample densities and speed ratios. The main plot shows $\rho v$ for the first tile on the slow (-) side vs. the same quantity for the first tile on the fast (+) side for several independent datasets. For both low density (filled symbols) as well as higher-density datasets (open symbols) Eq. (6) holds. This is also demonstrated in the inset, which shows $\langle \rho v \rangle_{\text{slow}}/\langle \rho v \rangle_{\text{fast}}$ vs. $\langle v \rangle_{\text{slow}}/\langle v \rangle_{\text{fast}}$ for the same datasets. Error bars show s.d. of the low average cell density (OD = 1), it is clear that $\rho v(x) = \text{constant}$ to within one standard deviation, which directly verifies Eq. (3).

**Experiments with $\beta(I)$ dependency.** Interestingly, experiments using low light intensities (which gave low swimming speeds) proved less successful, because at very low intensities we found a noticeably higher percentage of non-motile cells in the sample.
Fig. 5 Response of bacteria to the imposition of a 1D square wave with 270 µm wide stripes. a. The evolution of the speed profile normalised to its (time) average, with time increasing from red to violet (legend gives t in minutes): initially illumination is uniform, with the intensity getting increased from low to high at t = −7 min, then the pattern is applied at t = 0 for 15 min. b. The spatial profiles of various normalised quantities as indicated in the legend after 15 min of patterned illumination. Error bars represent s.d. Dashed line (and grey area) is the average (±1 s.d.) over all tiles for the uniform case (t < 0). Error bars show propagated s.e.m.

Discussion

Equation (3) is one of only a handful of exact predictions to date on the statistical mechanics of active particle systems. Its ‘weak’ form, for non-interacting systems was derived for RTPs\(^8\), while its ‘strong’ form was later derived both for RTPs\(^8\) and ABPs\(^9\). Taken together, our experiments using stepped and stripped light patterns give strong evidence that Eq. (3) holds at 1 ≤ OD ≤ 8 (0.15% ≤ φ ≤ 1.2%) for smooth swimming \(E. \ coli\) whenever we can be sure that steady state has been reached, either in the vicinity of \(x = 0\) in the stepped pattern or throughout the stripped pattern. Our swimmers are interacting throughout our concentration range\(^2\), even though collective motion is not observed until OD >> 10. Thus, our results verify the ‘strong’ form of Eq. (3) for ABPs.

The qualitative validity of Eq. (3), viz., that cells gather where they swim slower, or, equivalently for our cells, where the light intensity is lower, has already been assumed and utilised in recent work deploying such cells in smart (or reconfigurable) templated self assembly, or ‘painting with bacteria’\(^10,11\). Indeed, in a recent demonstration of how to perform bacterial painting with multiple shades of graded intensity levels\(^11\), there was attempt at checking the correctness of Eq. (3) en passant, which, however, was unsuccessful because of a high number of non-motile cells and the long stopping time of their strain, the latter producing memory effects. Our success in verifying Eq. (3) shows that carefully quantifying and subtracting the non-motile fraction and the use of a strain of bacteria with very short stopping time are essential ingredients in such an experiment. Indeed, without careful design most ‘real’ active systems are likely to display dynamic behaviour that is too complex to fulfil the assumptions leading to Eq. (3), as evidenced by our findings for the wild type strain with illumination-dependent tumbling rate. Note, however, that ‘smart templated active self assembly’\(^10\) using photo-activated swimmers is possible for any relationship of \(p−v\) in which the increase in one variable necessitates the decrease in the other.

Our experiments would not have been possible without spatially resolved differential dynamic microscopy (sDDM), which can reliably quantify swimming speed and relative density (along with many other parameters) over a wide range of length scales and cell concentrations. Its adoption can therefore provide new insights into a variety of systems displaying spatially varying dynamics, from biological taxis\(^26\) to collective motion.

Throughout, we have focussed on steady-state effects, although the consideration of time dependence proved crucial in interpreting apparent systematic deviations from the prediction of Eq. (3) for imposed stepped intensity patterns. Time-dependent effects are, of course, interesting in their own right. Thus, the response of active particles to a time-dependent topographic landscape that is self-assembled by the cells themselves\(^9\) has yet to be explored experimentally. On the other hand, it has recently been suggested theoretically\(^28\) and demonstrated experimentally\(^29\) that travelling-wave light fields can be exploited for transporting and rectifying light-activated swimmers. Exploitation of these and other opportunities should open up new fields of fundamental studies and applications.

Methods

Strains and sample preparation. We constructed three different strains of \(E. \ coli\) using plasmids expressing SAR86 γ-proteorhodopsin (a gift from Jan Liphardt, UC Berclay). These strains were designed to exhibit a fast response to changes in light intensity. This was achieved by deleting the unc gene cluster, so that the F\(_{1}\)F\(_{0}\)-ATPase membrane protein complex cannot work in reverse in the dark to generate proton motive force for swimming. The detailed molecular biology and strain characterisation have been reported before\(^10\). AD4 is a WT (run-and-tumble) swimmer derived from AB1157, whereas DM1 and AD10 are smooth swimming strains derived from RP437 and AB1157, respectively (see Supplementary Table 1). The two smooth swimming strains behaved similarly, although AD10 achieved a much higher swimming speed than DM1 and was also more efficiently powered by light. Therefore we mostly used AD10, with some additional data acquired using DM1.
Overnight cultures were grown aerobically in 10 mL Luria-Bertani Broth (LB) using an orbital shaker at 30 °C and 200 rpm. A fresh culture was inoculated into 1:100 of overnight grown cultures in 35 ml tryptone of LB (TB) and grown for 4 h to an optical density of OD600 = 0.2. The production of proteorhodopsin (PR) was induced by adding arabinose to a concentration of 1 mM as well as the necessary cofactor all-trans-retinal to 10 μM to the growth medium. Cells were incubated under the same conditions for a further hour to allow protein expression to take place and then transferred to motility buffer (MB: pH = 7.0, 6.2 mM K2HPO4, 3.8 mM KH2PO4, 67 mM NaCl and 0.1 mM EDTA). Single filtration (0.45 μm HATF filter, Millipore) was used to prepare high density stock solutions (OD = 8) which were diluted with MB to the desired cell concentration.

The samples were loaded into commercial 2 μl sample chambers (SC-20-01-08-B, Leja, NL) of dimensions 6 × 10 mm × 20 μm, where cells predominantly swim in the (x, y) imaging plane, but have enough room to ‘overswim’ each other in all three spatial dimensions. The chamber was then sealed using vaseline to stop air infiltration in the (x, y) field, low-magnification movies, one extracts the power spectrum of the difference between pairs of images delayed by time τ, g(ϕ, τ), where ϕ is the spatial frequency vector. Under suitable conditions and for isotropic motion, the intermediate scattering function f(qt), the qth moment of the density autocorrelation function, is given by

\[ g(q, t) = A(q)[1 - q^2] + B(q) \]  

(11)

Here, B(q) relates to the background noise and A(q) is the signal amplitude. Fitting g(q, τ) to a suitable swimming model of E. coli yields four key motility parameters: the mean speed of the sample (v), the width of the speed distribution (σv), the motility fraction β(q) of the diffusional coefficient of non-motile cells D(q), as a function of q. All of these should, ideally, be q-independent. In practice, there is some q-variation. We typically averaged the fitting parameters over 0.5q<2.2 μm−1 to give, e.g. ϕ = (vϕqτ) and β = βϕqτ.

In a diffusive system the structure function Φ(q) ≈ 1, A(q) is proportional to the same density33, and can therefore be used to determine relative densities by ρf/ρn = (A(q)/A(0))1/10. Note that ratioring the A(q) removes their strong q-dependence. Spatially resolved DDM (sDDM) is in principle straightforward: the above algorithm simply needs to be implemented on p × p pixels23 sub-movies. In practice, care is required in choosing the minimum p for which meaningful results can be obtained. We do this by illuminating a field of cells uniformly, measuring (v, ϕ, p, r, ν′) from individual p × p tiles in the steady state, and obtaining the probability distribution of these parameters. Under our imaging conditions, we found that these distributions became p-independent when p ≥ 64. We therefore chose p = 64, corresponding to 90 μm in the sample (see Supplementary Note 1 for details).

A full 512 × 512 movie yields g(q, τ) at 512τ/2 = 256 distinct q values. We divide it into 64 sub-movies of size 64 × 64 (pixel)2. This yields 8 × 8 × (64/2) = 2048 g(q, τ) to be fitted for each sub-movie vϕqτ, βϕqτ and τϕqτ/ρn = A(q)/A(0), where A(q) is measured from the same sample under uniform illumination (i.e. just before switching to a structured light pattern). These were averaged over 0.5 q<1.5 μm−1. The upper q limit is somewhat lower than what is typical for whole-movie analysis13 due to non-systematic failure of fitting at higher q values, presumably due to noise or windowing artefacts35.

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Author contributions
J.A. and V.A.M. contributed equally to this work. W.C.K.P. initiated the work. T.P. and A.D. designed mutants constructed by A.D. J.A. and V.A.M. performed experiments, analysed and interpreted data with T.P. and W.C.K.P., and wrote manuscript with W.C.K.P.

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