How blebs and pseudopods cooperate during chemotaxis

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Summary of new features in QuimP11

With the current paper we announce a new version of QuimP (QuimP11), freely available for download at *www.warwick.ac.uk/quimp*, and containing the following improvements. Multiple cells can be tracked in parallel, with an implementation of interacting active contours, as proposed by Dufour *et al.* (1). Segmentation is semi-automated, providing the tools to manually correct errors, including interactive setting of parameters globally or for individual frames.

Our Electrostatic Contour Migration Method (ECMM) (2) is utilised for tracking, and has been improved to give smoother, more robust results. ECMM now constructs fields using line charges (instead of discrete point charges). ECMM is also employed in sampling cortical fluorescence.

QuimP11 is bundled with scripts for MATLAB (©2011 The MathWorks, Inc.) that provide a framework for import and analysis of QuimP output.

For comprehensive details of QuimP11, its application to blebbing in *Dictyostelium*, and other applications, see thesis by Tyson R A (Richard Anthony) (2011) The cartography of cell motion. PhD thesis, University of Warwick (http://webcat.warwick.ac.uk/record=b2581468~S1).

ECMM: Migrating markers

Charged chains of line segments form the electric field in which markers migrate. A finite line *L* is defined in two dimensional Euclidean space by the endpoints s_1 and s_2. The vector $\vec{r}$ points from s_2 to the field position, *p*, and $\vec{r}_+$ points from s_1 to *p* (SI Fig. S1a). The electric field, *E*, at *p*, as described by Rowley (3), is given by

$$\overrightarrow{E(L,\lambda)} = \frac{k\lambda}{d}(\hat{r}_+ + \hat{r}),$$
$$d = \frac{2L}{(r_+ + r)^2 - L^2},$$

where *k* is the electrostatic constant, λ the magnitude of the charge on *L*, $\hat{r}$ and $\hat{r}_+$ are unit vectors of $\vec{r}$ and $\vec{r}_+$ respectively. A marker, positioned at *p* within an electric field, and carrying a single unit of positive charge, will experience a force, $\vec{F}_p$, equal to the sum of the forces contributed by each line of charge,

$$\vec{F}_p = \sum_{i=1}^{U} \overrightarrow{E(L_i,\lambda)} + \sum_{j=1}^{V} \overrightarrow{E(L_j,-\lambda)}\,\overrightarrow{E(L_j,-\lambda)},$$

where *U* is the number of positively charged lines and *V* the number of negatively charged lines. The force $\vec{F}_m(p,q)$ experienced by a marker *m* (of zero mass), at *p*, carrying a charge of magnitude *q*, is given by

$$\vec{F}_m(p,q) = \vec{F}_p.q.$$

Markers are migrated through the field numerically, using the explicit Euler method, by solving the equation

$$\overrightarrow{p_{t+1}} = p_t + \vec{F}_m(p,q).\Delta t.$$

ECMM: Mapping outlines - simple test case

A cell outline, Γ, consists of a chain of connected nodes at a density of ω^N (nodes per pixel) defining the resolution of the segmentation. Markers and line charges are placed at densities independent of ω^N, and of one another, to control field complexity and mapping resolution. We first consider a simple test case consisting of two, non-intersecting, outlines (SI Fig. S1c,d).

Endpoints defining line charges are spaced equidistantly along the contour of Γ^T at a density ω^L, typically equal to, or less than, ω^N. This defines the complexity of the electrostatic field. Similarly, markers are placed equidistantly along Γ^T at a density ω^M, which is typically larger than ω^N. This defines the resolution of the mapping.

In the first case (SI Fig. S1b) positively charged markers are migrated out-to-in, densities being equal ($\omega^N=\omega^L=\omega^M$), to produce a smooth mapping

irrespective of large deformation. Numerical accuracy is controlled by the time step used in the numerical procedure. The ratio of charge magnitudes can be used to alter the uniformity of the mapping.

In the second case (SI Fig. S1c) a higher resolution map is computed (ω^M is increased), but the extra computational cost is offset by lowering the field complexity (ω^L is reduced). This is possible as altering ω^L does not significantly alter the force on markers, and a complete, smooth field is still present. Importantly, markers are able to cross the boundaries of line charges by simply switching their polarity.

ECMM: Mapping cell outlines

We refer the reader to the thesis by Tyson R A (page 45) for the complete scheme, and only make key observations here. When mapping cell outlines, outlines are overlaid in order to compute points of intersection. These points define *sectors*, used to disassemble the mapping into pieces. Identifying the configuration of sectors is key to avoiding severe marker dilation and/or compression (SI Fig. S1g). ECMM ensures that the longer of two edges is always mapped to the short edge, regardless of their temporal order. This is referred to as the Forward/Reverse scheme (F/R scheme). This also avoids having to reinitialise markers for the next time point to achieve homogenous spacing.

As field lines never cross this ECMM is very robust. This approach is equivalent to solving problems in hydrodynamics where an incompressible fluid is driven by a potential. Analogously, the density of markers is kept constant in our method, while they are migrating from one contour to the other. This constraint, along with fast computation, makes ECMM a strong competitor amongst other methods such as level set methods which have been suggested for tracking cell contours (4,5,6,7).

As cells move by rear contraction and front extrusion, rather than translation, our mapping scheme captures local deformation normal to the membrane, assuming no lateral membrane/cortical movement, which schemes such as that by Driscoll *et al*. (8) attempt to model. In our method protrusions at the front to do incur lateral movements at the rear.

ECMM: Tracking markers over sequences of outlines

ECMM output allows any arbitrary position on a cell outline to be tracked over any number of frames. Previously, this was approached by labelling markers with unique identifiers and building a lineage. However, lineages become broken if markers are removed (typically to maintain marker homogeneity), and only allow tracking of discrete points with no indication of their position in the chain.

Here, we instead use a system of decimal values and linear interpolation. Outlines are normalised to length 1, and markers assigned *decimal positions* (*DP*s) in the range *(0,1]* according to the distance around the circumference from an arbitrarily chosen head marker. Each marker also has a *marker origin* (*MO*) indicating the position on the previous outline to which it is mapped. *MO*s are set as duplicates of *DP*s in cases where markers are mapped forward in time (in reference to the F/R scheme), or are interpolated values from neighbour *DP*s when mapping in reverse. See SI Fig. S2 for a worked example.

Given *DP*s and *MO*s on each outline, one can use linear interpolation to track any position in the range (0,1] according to the computed mappings, irrespective of the mapping resolution, over all time points.

QuimP11 software overview

QuimP is written in the Java programming language as a series of plug-ins for the open source image processing tool, ImageJ (9). The BOA plug-in provides a supervised image segmentation algorithm, in the form of an active contour, for extracting cell outlines. The resulting outlines are fed to the ECMM plug-in for boundary tracking. Cortical fluorescence intensities are sampled by the ANA plug-in in which the user defines a cortex width. Finally, the QAnalysis plug-in compiles spatial-temporal maps of motility, fluorescence, curvature, and tracking.

QuimP uses an active contour model for segmentation (10). A chain of nodes is manually initialised around a cell on the first time point by drawing a shape with the computer mouse. The chain is a physical representation of a visco-elastic rubber-band with forces acting on each node. Internal elastic forces make the chain constrict and approach the cell boundary in an iterative manner. The local contrast increases when the chain approaches the cell boundary. From the local contrast an opposing external force is computed such that internal and external forces balance out one another when the chain matches the cell boundary best.

Multiple chains may be initialised in parallel around cells that come into close proximity, preventing erroneous segmentation.

Earlier QuimP versions track nodes of the active contour model to compute cellular deformations (11,12), but this has been replaced by ECMM.

Computing cortical fluorescence

Quantifying cortical fluorescence can be problematic for several reasons. The cortex has a relatively loose definition in eukaryotic cells as an actin rich layer just below the membrane. Cortex thickness varies between cells, can be non-uniform, and is also effected by segmentation errors. To account for these issues, we define the cortical region as a continuous strip, of uniform

thickness (decided upon by the user), bounded by the cell outline. At uniform intervals on the cell outline, only the maximal intensity is recorded within the strip (3x3 pixel average).

The inner boundary of the strip is computed by iteratively shrinking the cell outline in the direction of the surface normal while maintaining node density and preventing self intersections in order to preserve thin projections (SI Fig. S1f). Markers on the cell outline are then migrated across the cortex by ECMM, their path defining the cortical region associated with a position on the outline. The maximal sampled intensity is recoded for each marker and normalised to the mean cytoplasmic intensity (optional) (SI Fig. S1g).

Automated Protrusion Tracking

Locating projections is done in three steps; 1) maxima are identified in velocity maps; 2) beginning at maxima, deformations are tracked forward and backward in time; 3) the resulting paths are trimmed and/or joined according to their relative positions.

In sequences where segmentation noise and membrane fluctuations occlude the location of slowly expanding protrusions (as in our case when using high frame rates) we take the additional step of computing an integrated velocity map. Membrane velocity is integrated over time (Δt=2 seconds) along tracks computed by ECMM (see Main Text Fig. 1c,d,e).

Regional maxima of integrated membrane velocity are located by applying the extended *h*-maxima transform (13) (height threshold, *h=0.3* μm). The transform results in regional peaks, as opposed to singular local peaks, hence identifying just one peak per projection, when in reality many local peaks may exist. A value of h = 0.3 μm/second was found to suppresses background noise while keeping regional peaks for consecutive projections separate.

Velocity maxima could be defined most simply as the weighted centre of regional peaks regions. However, this can skew the result away from the centre of deformation, particularly in cases where projections in very close proximity become incorrectly merged (SI Fig. S3, projection 12). Our chosen alternative is to compute a weighted centre through time only (vertically on maps), and then to locate a velocity maximum at the maximum at that weighted time point (within the bounds of the regional peak). Missing peaks can simply be added manually if required.

Beginning from these maxima, projection paths are traced out by following ECMM tracks backwards and forwards in time while the integrated velocity remains above 30% of that at the maximum (SI Fig. S3b). In cases where projection paths merge (lie within a threshold distance spatially and temporally) paths must be shortened. This occurs when the 30% *of peak velocity* cut-off during path tracing is insufficient to separate projections that emerge in quick succession, at the same position (such boundaries can be obscured in integrated velocity maps). Intuitive rules are used to automatically

trim and join projection paths where appropriate (see thesis by Tyson R A (page 39)).

An effect of computing integrated velocities is that two blebs in close spatial proximity can sometimes appear as a single regional maximum (< 3% of all cases). In that case only the faster of the two blebs will be counted. To validate that the tracking of this bleb is valid, paths are plotted back onto the original sequence for inspection (SI Fig. S3c). For tracking results see SI Videos S2, S4, S5, S8.

Projection classification

Manual classification of membrane projections as blebs was based on the detachment of membrane from the cortex leaving behind an actin scar and a clearly visible gap between the scar and the detached membrane. A concomitant drop in the actin label at the detached membrane was observed in most cases, particularly enhanced in fast blebs. If a drop was observed it was always followed by a subsequent build-up of actin in the detached region. If a bleb nucleated on top of an existing bleb without a resolvable speed separation (either due to true seamless extension, or being obscured by noise or limited frame rate) then they were classed as *stacked-blebs*. Stacked blebs are included in all statistics unless otherwise stated (Developed cells under 0.7%, $N^{\text{stacked-blebs}}=1$; developed cells under 2.0%, $N^{\text{stacked-blebs}}=13$; vegetative cells under 2.0%, $N^{\text{stacked-blebs}}=0$).

Blebs were classified as blebbopods if their traces included a subsequent extension associated with increased actin. Actin based protrusions were identified as advancing regions in which actin stayed clearly associated with the membrane.

If a classification could not be discerned the protrusion was labelled as *unclassified* and excluded (Developed cells under 0.7%, $N^{\text{unclassified}}=21$; developed cells under 2.0%, $N^{\text{unclassified}}=7$; vegetative cells under 2.0%, $N^{\text{unclassified}}=25$).

Testing for cell contraction simultaneous with blebbing

To test if contraction is simultaneous with bleb expansion, we took high magnification (100x), 50 sec sequences of developed Ax2 cells under 2% agarose cells, acquired using soluble GFP to strongly label the cytoplasm to allow fast acquisition rates (10fps). High speed sequences are required to accurately capture the time of bleb nucleation and bleb halting. Cells were chosen exhibiting bleb expansion in absences of other membrane projections around. Sequences were intensity homogenised and Gaussian smoothed ($\sigma=$ 0.13 μm) and images of cells binarised using intensity thresholding. Binary images were used to visualise and compute imaging plane changes in area. Regions of area gain were computed by subtracting the binary image at the

time of bleb halting from that of bleb nucleation, and *vice versa* for loss. See SI Fig. S8 and SI Video S7 for results.

As cells are flattened under agar, the imaging plane cell area provides an improved estimate of cell volume relative to other conditions. However, detailed investigations would require 3D data.

Average circular statistics

We computed polar plots for average membrane velocity, curvature, and actin fluorescence. Average membrane velocity around developed cells chemotaxing under 0.7% agarose (SI Fig. S6a) shows a positive peak in the direction of the gradient, and a negative peak at the rear, reflecting net translocation towards the chemoattractant. At the front we observe a large variation reflecting cycles of actin based protrusion which is not as pronounced at the flanks. Average curvature at the front is positive and correlates with the distribution of protrusions and where blebs are infrequent. We do not see average negative curvature at the flanks, as would be suggested by our observation of bleb nucleation flanking pseudopodia. This is expected as blebs are both less frequent, and once formed, have positive curvature by definition. In general, blebs are occluded in these representations due to their short-livedness making them problematic to study using average statistics.

Average GFP-ABD label levels around cells are skewed towards the rear, reflecting our observations by eye that the label is most dense in the uropod in cells under 0.7% agar, as similarly found in *Dictyostelium* and blebbing melanoma cells (14). Cells chemotaxing under buffer show this same fluorescence distribution ruling out effects of imaging under agarose (SI Fig. S6d). To what extent this represents the true distribution is undetermined as presumably GFP-ABD labels older F-actin and rear contraction may be acting to concentrate the label at the rear.

Cells chemotaxing under 2.0% agarose (SI Fig. S6b,c) are rounder and have distinct positive curvature at the leading edge. This is indicative of the leading edge being advanced by blebs (generating high positive curvature). Detailed analysis of individual blebs, however, revealed the bimodal distribution of their nucleation (Main Text Fig. 1), hence they do not nucleate directly at the leading edge. Only 3% of blebs emerged directly on top of previous blebs.

Computing distributions of normalised curvature and statistical testing

To test if curvature is the sole contributor to bleb nucleation we constructed frequency distributions using normalised curvature. For example, in cases where cells are rounded (having no negative curvature) we would expect blebs to form at regions of lowest positive curvature, an affect which is masked in our previous analysis of bleb curvature distributions (Main Text Fig.

3). Similarly, where several regions of negative curvature exist, one may expect a bleb to form at the maximal negative curvature.

To test this hypothesis we computed the maximal and minimal curvature of a cell at the frame immediately prior to bleb nucleation and normalised this range to [0,1]. The curvature at the bleb site was then located in the normalised range and recorded into a frequency distribution. Similarly, a test distribution, representing the expected frequency assuming blebs occur at arbitrary curvature, was generated by repeatedly moving bleb tracks to random locations on the cell contour.

Observed and expected frequencies were compared in 8 classes (width 0.125) using Chi-squared tests and final *P* values corrected for multiple comparisons using Bonferroni correction. See SI Fig. S8 legend for results.

A mathematical model for bleb nucleation

To investigate how actin driven protrusions and blebbing are mechanically coupled we developed a mathematical model for bleb initiation where we look at the very first but critical event of membrane detachment only. We consider the 2D cross-section of a cell sandwiched by an agarose overlay. We take a modelling approach similar to (15,16,17) which is a discrete active contour model where the membrane is mechanically coupled to the cell cortex by tethers acting at as linear springs. Tethers are assumed to break when extended past a threshold length. This leads to irreversible local detachment of the membrane. The full model accounts for active deformation of the cortex, but here we ignore the temporal evolution of the cortex altogether, as on the timescale of bleb initiation actin scars remain relatively static. We define an energy functional for the membrane with the contour length normalised to 1. It is determined by internal energies due to membrane tension and resistance to bending, and external contributions due to coupling to the cortex and hydrostatic pressure (Equation 1).

Equation 1

$$E_{membrane} = \oint_0^1 \left(E_{tension} + E_{bending} + E_{coupling} + E_{pressure}\right) ds$$

A gradient-descent method is used to numerically minimize $E_{membrane}$ using finite differences to approximate the first and second order terms involved in membrane tension and bending energies (10).

Equation 2

$$E_{tension} = \frac{1}{2} \propto \left\| \frac{d(x(s) - x_0)}{ds} \right\|^2$$

Equation 3

$$E_{bending} = \frac{1}{2}\beta \left\| \frac{d^2x(s)}{ds^2} \right\|^2$$

Equation 4

$$E_{coupling} = \frac{1}{2}k \left\| \frac{d(L(s) - L_0)}{dl} \right\|^2$$

Equation 5

$$E_{pressure} = \Delta p$$

where α is membrane tension, x_0 the membrane resting length, β the bending rigidity, k the linker stiffness, L the current linker length, and L_0 the default linker length.

The protruding membrane experiences a drag force. We consider a Stokes frictional coefficient, Γ, for a spherical object moving in a viscous fluid,

$$\Gamma = 6\pi\eta R$$

,where η is the dynamic viscosity of water at 20°C ($1x10^{-3}$ N s/m^2) and R the sphere radius, here estimated as the average radius of blebs formed over the simulation ($1.15x10^{-6}$ m).

Our model is parameterised as presented in SI Table S1. However, aside from being two dimensional, it must be appreciated that parameters that determine tension in a simulated chain of springs have no true life counter parts or cannot be resolved with current experimental techniques. For example, apparent membrane tension is the sum of the tension in the bilayer and adhesion energy at the membrane-cortex interface, and these are generally not separable in tether-pulling assays as well as magnitudes varying between cells and conditions (18). Furthermore, our model does not individually account for the components that determine tension, such as membrane-protein content (18), membrane unfolding (although limited in *Dictyostelium*) and roles of the endocytic cycle (19). Particularly problematic is the simplification of membrane-cortex adhesion, for which we use a low density of linkers to simulate what is in all likelihood a highly dynamic molecular interaction. Linkers also limit the propagation of surface membrane tension around the simulated cell.

The cell radius was set to the observed average in cells under 2% agar. The default linker length, L_0, was estimated from the spacing between integrins and cortex in cell adhesions, 40nm (20). We then defined the resting length of a membrane segment, x_0, as being 95% of the average length (assuming membrane elasticity, determined at 2-3% (21), is the overwhelming factor at the time scale of blebs). Bending rigidity was set to well documented magnitudes obtained from vesicles, in the order of 0.1-0.2 pN/μm (18).

Tension was then introduced into the initial system by tuning the membrane stiffness coefficient to achieve tension measured in tethers pulled from talin null *Dictyostelium* cells, 0.8 pN/µm (22). The coefficient is in the same order as that used in other models (Strychalski, 2-10 pN/micron)(16). To create blebs, static pressure was set between 17 pa and 110 pa (23, 24) and linker breaking length determined through numerical simulations.

SI Video S9 shows the complete simulated cell where outward directed forces acting on the cortex have been used to simulate the formation of a pseudopod (*t=0*). On the timescale of bleb expansion (<3 sec) we treat the cortex as rigid which is in line with our observation (Main Text Fig. 1c), and that of others (25), namely that during that event the cortex stays in place and shows no signs of deterioration. At *t*=0 the system is in equilibrium and tethers are homogeneously distributed to rule out any effects of membrane curvature on tether distribution. At *t*=0 tethers are allowed to break and they do so in the concave region at the base of the pseudopod and the membrane detaches by unzipping. The drop in intracellular pressure is not sufficient to stop further unzipping. This is supported by our experiments, as if it were and pressure was relaxed, we would not see nucleation of blebs in quick succession. We also would not observe continuous unzipping, similar to what has been described as circus movement. In order to stop further unzipping we increase the effective spring constant on the two basal tethers, by multiplying the default spring constant by 0.5**d*/*a*, where *d* is the distance between the basal tethers, and *a* is the average distance between tethers. The assumption here is that in the real 3D situation the neck region is obviously not held back by only two tethers, but also tethers distributed along the entire base. Diaz-Munoz et al. movie video S3 (26) resembles, for example, continuous unzipping that we observed in our model prior to introducing simulation of a continuous base. As our focus is on bleb nucleation this detail does however bear no relevance for the main conclusions of the current paper. The simulated bleb reaches 99.9% in size after 0.70 seconds.

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SI Figure Legends.

Figure S1. Test case for marker migration using virtual line charges. **(a)** Quantities for computing the force experienced at p as a result of charge L (Adapted from Rowley, 2006 (3)). **(b)** A smooth, continuous force field generated by two lines of opposing charge. **(c)** Contour Γ_T is mapped to Γ_{T+1}. Depicted nodes mark endpoints of line charges. Markers (point charges) are placed on Γ_T, matching line charge density, and migrated until Γ_{T+1} is reached (black paths). **(d)** The smooth nature of the field allows line charge resolution to be lowered, saving computational time while maintaining valid mappings for arbitrary marker resolution. **(e)** Example mapping between two cell contours. **(f)** ECMM mapping applied to sampling maximal fluorescence intensity within the cortex. The cell outline is shrunk continuously inwards in the direction of the surface normal, resulting in an inner boundary (green) marking the cortical region. The chain is self-interacting preventing edges from intersecting. Pixel intensities (3x3 averages) are sampled along marker paths and the maximum recorded. **(g)** A complete mapping of virtual markers and the direction of the migration as used in the *F/R scheme*. See SI Text S1 for details.

Figure S2. ECMM marker tracking using decimal positions. **(a)** Decimal position scheme for labelling markers; Y_T is normalised to length 1, and decimal positions (DPs) assigned in the range (0,1], clockwise, relative to a arbitrarily chosen head marker. **(b)** Outlines Γ_T and Γ_{T+1} represent a low resolution segmentation of a cell moving south. Markers are migrated in accordance with the *F/R scheme* (SI Fig. S2). At the rear, markers are migrated forward in time ($Y^m = Y_T$), and marker origins (MOs) are set equal to marker DPs. Markers are then assigned new DPs for their positions on Γ_{T+1}. At the cell front markers are migrated in reverse ($Y^m = Y_{T+1}$). MOs are assigned by interpolating the DPs of markers on Y_T . Using interpolation, the record of DPs and MOs allows any arbitrary position on an outline to be tracked over any number of frames.

Figure S3: Protrusions and blebs are simultaneously located and tracked automatically by computing an integrated velocity map to reduce noise, identifying velocity maxima, and following ECMM computed tracks over the period of deformation. **(a)** Integrated velocity map of cell shown in Main Text Fig. 2c. Velocity maxima are indicated by circles. **(b)** Beginning at velocity maxima, projections are traced forward and backwards in time (vertically) according to ECMM, while velocity remains above 30% of the peak speed. Projections in close spatio-temporal proximity can become merged into a single peak (here projection 11 is missed), however by design this does not result in an incorrect tracking of projection 12. Projection 11 can be included manually by adding a peak and allowing the software to trace it. If overlaps occur, traces may be joined or trimmed, for example pseudopods 4 and 5 are constructed from two peaks. **(c)** Projection tracks overlaid onto the image sequence for classification (SI Video S2).

Figure S4. Determining peak projection speed from displacement profiles. **(a-b)** Motility profiles of a bleb and F-actin driven protrusion respectively. Noise prevents accurate estimation of peak speed, defined either as the maximal

profile speed (black dashed line), or the maximal of a 3 frame average (red dashed line). **(c-d)** Peak speed estimated by fitting a sigmoid curve to displacement profiles (see SI Text S1), and determining the maximum gradient. **(e)** Plot of manual versus ECMM-APT determined peak projection speeds. Projections detected by ECMM-APT were matched visually with a manually created set (traced directly from raw image frames). Data points on the diagonal indicated exact agreement. The mean relative error of the automated method was 0.18.

Figure S5. ECMM-APT analysis examples. Cellular deformation was analysed with our custom software for tracking projections. The centre of projections (peak velocity) is determined and subsequently tracked through time using our ECMM mapping until falling below a threshold velocity (see SI Text S1 for details) **(a)** Analysis of vegetative cell chemotaxing towards folate under 2.0% agar. Detected bleb are overlaid (black). At *t*=43 sec the cell establishes a new front at which blebbing is frequent (see SI Video 4). **(b)** Analysis of blebbing Fundulus deep cell (courtesy of Rachel Fink, Mount Holyoke College) shows blebs nucleating at regions of negative curvature. Two vertical stripes of extended negative curvature indicate the cell's *waist*, surrounding the rear, where active protrusion is suppressed during directed migration (see SI Video 8).

Figure S6. Time-averages of relevant parameters of blebbing *Dictyostelium* cells under agar as visualised by polar plots. The leading edge is defined as the marker most advanced in the cAMP gradient at each time point. Locations around the cell perimeter are encoded counter clock-wise as normalized distances from the leading edge (0,1]. **(a)** Average membrane integrated velocity (0.7% agar, [25;75] percentiles) shows expansion at the leading edge and contraction at the rear. Despite their high velocity, blebs are obscured due to their short duration. Similarly, cell poles show positive curvature on average, while flanks are relatively flat. Specific curvatures related to blebbing are lost when averaging over time. Cortical actin is enriched at the cell rear. Fluctuations at the leading edge are due to protrusion dynamics superimposed onto the global distribution. **(b-c)** Under 2% agar cells are more rounded in shape and show high curvature due to bleb formation. **(c)** Cell chemotaxing towards cAMP under buffer has an asymmetric distribution of actin concentrated at the rear (GFP-ABD label).

Figure S7. High speed movies of developed Ax2 cells under 2% agarose show continuous contraction during bleb expansion (soluble GFP at 100x magnification, 10 fps). 10 examples of blebs (expanding in absence of other membrane deformations around the cell) were analysed for global and local changes in the imaging plane area (see SI Text S1 for methods). **Top**: A series of blebs propagate along the top edge resulting in a global increase in area of 2.32 μm^2. However, area gain (green) is isolated to the blebbing region and is in excess of 9 μm^2. The remaining periphery contracts resulting in an area loss (red) of 6.8 μm^2. **Bottom**: Further examples of gain-loss maps. On average, cells increased in area by 0.81% (±0.64), gained 2.72% (±1.45), and lost 1.91% (±0.98) (N=10). See SI Video S7. Scale bar 5 μm. (±SD).

Figure S8. Normalised curvature analysis shows blebs to nucleate significantly more often at the lowest measured curvature, but not exclusively. At the frame immediately prior to bleb nucleation, cell curvature was mapped to the normalised range [0,1] and the curvature at the bleb site located within this range. This was repeated for all blebs to generate a normalised curvature distribution. Similarly, bleb tracks were randomly repositioned to generate a test distribution. See SI Text S1, "Computing distributions of normalised curvature" for method details. Distributions were compared using Chi-squared tests in classes of width 0.125 and Bonferroni correction.

SI Video legends

Video S1. Ax2 cell under 0.7% agar imaged with an actin label (GFP-ABD) and negative stain (RITC-Dx) for segmentation. The cell exhibits frequent blebbing at its flanks. Blebs can be seen to nucleate at the base of previous blebs where negative curvature is induced.

Video S2. ECMM-APT analysis of blebbing developed AX2 cell under 0.7% agar, as shown in Main Text Fig. 2 and SI Fig. S3 (GFP-ABD label). The cell was segmented (red outline) and projections detected (white paths). Example projections marked in Main Text Fig. 2 are labelled *p.1*, *p.2*,*b.1-b.3*, *c1*.

Video S3. ECMM-APT analysis of blebbing developed Ax2 cell chemotaxing under 2.0% agar as shown in Main Text Fig. 4d (GFP-ABD label, cAMP source at bottom, 2fps (x3)). The cell was segmented (red outline) and projections detected (white paths). At 16.5 sec, blebs are seen to propagate from the front and along the cell flanks (bottom to top).

Video S4. ECMM-APT analysis of vegetative Ax2 cell under 2.0% agar, as shown in SI Fig. S5a (GFP-ABD label). The cell was segmented (red outline) and projections detected (white paths). The cell changes direction 180 degrees with blebs forming at regions of negative curvature at the new front.

Video S5. ECMM-APT analysis of blebbing developed Ax2 cell under 0.7% agar (GFP-ABD label). The cell was segmented (red outline) and projections detected (white paths).

Video S6. Examples of filopods observed in Ax2 cells chemotaxing under 0.7% agar (2fps). In 25 vegetative cells (37 min) we observed 6 bleb-filopod interactions. In 24 developed cells (36 min) we observed no bleb-filopod interactions. **Top left**: vegetative cell, bleb expands over a filopod. **Middle left**: vegetative cell, bleb expansion halts at the base of a filopod. **Bottom left**: vegetative cell, filopod disassembles. **Top right**: vegetative cell, bleb expansion halts at the base of a filopod. Scale 2 μm. **Middle right**: vegetative cell, at 7 sec we observe the only case where bleb nucleation could have occurred due to the presence of a filopod (scale 5 μm). **Bottom right**: Developed cell (5.5 h), blebbing without any visible filopods (scale 5 μm).

Video S7. High speed movies of developed Ax2 cells under 2% agarose (soluble GFP at 100x magnification, 10 fps). Cells show continuous contraction during bleb expansion. See SI Fig. S7 for analysis. Scale bar 5 μm.

Video S8. ECMM-APT analysis of blebbing Fundulus deep cell (phase contrast, courtesy of Rachel Fink, Mount Holyoke College). The cell was segmented (red outline) and projections detected (white paths). For motility maps see SI Fig. 5b.

Video S9. Simulation of bleb initiation as a result of negative membrane curvature, as shown in Main Text Fig. 4. The cortex (green) is fixed and is

coupled to a freely moving membrane (red) by linkers (black). Bleb like membrane structures nucleate spontaneously at regions of negative curvature. For display only, the cortex has been shrunk by 0.3 microns to make linkers visible.