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Tools for analyzing cell shape changes during chemotaxis

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Summary

Chemotaxis refers to the ability of cells to sense the direction of external chemical gradients and respond by migrating towards the source. A thorough understanding of the chemotactic response of amoebae and neutrophils requires careful quantification of the cell shape changes observed during cell movement. The stochastic nature of this response calls for a statistical characterization of cellular morphology and this requires the processing of large data sets. For this reason, automatic image analysis algorithms are highly desirable and are becoming increasingly available. These usually include a combination of techniques from image segmentation, morphological transformations, as well as the incorporation of numerical algorithms and physical models. Here we review recent developments in the tracking and understanding of motile chemotaxing cells, with a particular emphasis on the description of pseudopodial activity in chemotactic *Dictyostelium* cells.

Introduction

Chemotaxis, the ability to sense the direction of external chemical sources and respond by polarizing and migrating toward chemoattractants or away from chemorepellants, is crucial for proper functioning of single cell organisms, such as bacteria¹ and amoebae², as well as multi-cellular systems. Chemotaxis plays an important role in the immune system and in many aspects of development and tissue maintenance. Normal physiological processes such as lymphocyte homing, angiogenesis, embryogenesis, neurogenesis, and wound healing require accurate migration of specific cells. On the other hand, inappropriate regulation of chemotaxis plays a role in excessive inflammation and inflammation-related diseases such as asthma, multiple sclerosis, and arthritis³. Chemoattractants also direct cancer cells to sites of metastasis⁴.

For a cell to chemotax, a guidance system is needed to detect the location of the signal source and orient the cell accordingly. For locomotion, cells develop distinct leading and trailing edges, and this involves asymmetrical distribution of multiple proteins and lipids leading to internal cell reorganization and changes in cell morphology. The cell moves through a series of regulated cycles of pseudopod and lamellopod extensions and retractions which cause the cell body to translocate. These processes are all intimately coupled in a chemotactic cell and involve extensive and complex sets of molecular interactions. Owing to the high complexity of the networks that regulate directed cell migration, attempts to

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incorporate all aspects of chemotaxis in one comprehensive model seem daunting. For this reason, a view of chemotaxis has emerged that considers it as the product of several interacting but independent processes: gradient sensing, polarization and motility⁵⁻⁷.

Recent years have seen great progress in the integration of quantitative experimental methods and computational models to unravel the intricate networks that regulate cellular function. For chemotaxis, most of these models have focused on gradient sensing or polarization^{7, 8}. However, to understand directed cell migration, researchers must come to terms with the mechanism by which gradient sensing and polarization are tied to the cell shape changes that accompany motility. As the basic building block of eukaryotic motility is the pseudopod, we must explain how cells guide the extension and retraction of pseudopods⁹. This is complicated by the fact that pseudopodial protrusions during chemotaxis appear to be random events whose location or duration may be biased by the gradient sensing and polarization mechanisms. Thus, we need a statistical description of chemoattractant-induced cell shape changes. This calls for the analysis of large numbers of cells, pointing to the need for automated image analysis techniques. Here, we review several recently developed image processing techniques and algorithms that have been proposed for tracking and characterizing cell shape changes observed during motility. We also describe some of the new insights that have been obtained by these analyses.

Isolating cells from images: Segmentation

Before characterizing cellular deformations, any automatic image processing algorithm must first isolate the cell. A basis for all the tracking techniques described below is image segmentation: the division of a digital image into regions (segments) that, in this case, separates the pixels associated with cells from those of the background (Fig. 1). The specific algorithms used for segmentation depend on the imaging modality (e.g. fluorescence, phase contrast or differential interference contrast microscopy). Nevertheless, the methods can be broadly categorized into two classes.

The first class compares pixel values against predefined thresholds to isolate cellular regions (Fig. 1c). For fluorescent images, these schemes work well as the pixels inside cell are brighter than those outside. For non-fluorescent images, such as phase contrast or differential interference contrast (DIC) images, variations of thresholding are needed. In phase contrast images, pixel intensity differences arise from the anisotropic properties of the medium through which the light travels. Moreover, a “halo”-like phase ring is usually observed around the cell perimeter. While intensities inside the cell vary because of the numerous compartments distributed there, those outside the cell are considerably smoother. These differences can be used to detect the edges of cells by computing the pixel intensity gradient. The resulting segmentations will be quite noisy, but can be corrected using morphological operations. Cells imaged using DIC, because of the existence of a prism-induced shear direction, have both bright and dark edges, but little contrast between. Thus, direct edge detection or gradient mapping do not give satisfactory results for cell shape analysis. The line integration method, combined with pre- or post-processing such as deconvolution, Hilbert transform, or variation-based texture extraction can be used to transform DIC images to pseudo-fluorescent images^{10, 11}. In general, these methods are

straightforward and computationally efficient, but may be problematic for noisy images because these fluctuations can cause regions in the digital image to be above or below the threshold incorrectly and hence the cell shape is misidentified. Thus, before images are segmented, they are usually filtered to remove noise¹².

The second class of algorithms, known as active contour¹³ or deformable models¹⁴, finds features in the image by evolving a contour under the action of internal and external forces (Fig. 1d). The forces can be defined according to constraints on shape properties such as convexity and curvature, as well as from the distribution of the image intensity at the boundary. Algorithms in this class typically yield more robust segmentations but are computationally more expensive. Moreover, they require proper – usually manual – initialization.

One problem with any type of segmentation is the possibility that multiple cells come into contact and are grouped together (undersegmentation), or that a single cell is segmented into more than one object (oversegmentation). Additional information, either from spatial features or temporal correlations, is needed to avoid these segmentation errors, which make single-cell based shape analysis difficult. This is an area of active research in which algorithms that use information from multiple frames are being considered¹⁵.

Analyzing dynamic shape evolution during migration

Having isolated a cell in a sequence of images, it is possible to compute global aspects of the cell such as the area (or volume if three-dimensional images are available), centroid, eccentricity, etc. and characterize cell motility by the change in these parameters over time. One of the most comprehensive tools for doing this is the Dynamic Image Analysis System (DIAS, Soll Technologies), which is able to outline cells semi-automatically, determine movement paths of the cell center, and calculate a variety of variables to describe motility and morphology¹⁶. Most of these descriptors, such as velocity, acceleration, persistence, and roundness, provide global information about movement. A few of the others, such as positive and negative cytoplasmic flows, describe local information about the cell shape at each time point. Unlike the other software packages described here, DIAS has been used to analyze three-dimensional images. However, it does not yet provide an automatic means for detecting pseudopods, which must be delineated by hand¹⁷.

Machacek and Danuser used the level set method (LSM) to describe the protrusion of epithelial cells¹⁸. The LSM is a numerical technique for tracking interfaces and shapes that has been widely used in various fields including computer graphics, image processing, computational fluid dynamics, and material science¹⁹. In the LSM, the cellular boundary defines a higher dimensional potential function, known as the signed distance function, for which the boundary is the zero-contour (Fig. 2). Given two such potential functions (defined by the boundaries of two successive frames of a movie), an evolution equation (a type of partial differential equation known as the Hamilton-Jacobi equation¹⁹) can be defined for which the two potential functions are the initial and final cell outlines. This approach enables one to propagate the boundary markers in their normal direction between two consecutive frames to represent the local displacement over time. There are two advantages

to the implicit representation, and hence the LSM. First, the potential function is defined and evolved everywhere. This means that the computation can be done on a fixed rectangular grid, which is considerably simpler than techniques where the grid must change as the boundary's shape evolves. Second, it allows one to handle large deformations – even those involving topological changes, such as the appearances of holes. The main drawback of the LSM, however, is its high computational cost. To circumvent this problem, an alternative scheme was proposed in which adjacent membrane markers are assumed to be connected by linear springs, and markers at successive time points are connected by torsional springs. The positions of the markers, and consequently the protrusion directions, are defined by the equilibrium of the spring system. To deliver topologically consistent solutions, this method requires imaging with high spatial and temporal resolution.

The mechanical scheme described above is one of several techniques for characterizing cell shape changes by tracking boundary markers. Tyson and colleagues recently proposed a related algorithm, the Electrostatic Contour Migration Method²⁰ (ECMM). The ECMM assumes that there exists a fixed virtual electrostatic field, formed by placing positive or negative point charges evenly along the cell outlines of two consecutive frames (Fig. 3). This electrostatic field forces each boundary point to migrate with a magnitude and direction determined by the local field line along its path. To implement the scheme, cell perimeters at two different frames are superimposed. The two edges are then separated into distinct sectors between points where the two boundaries meet. Associated with each sector is part of the edge from each of the two frames. The virtual markers are placed on the longer of the two boundaries and evolve (following the electric field) towards the shorter one, irrespective of which is the earlier frame. The evolution from the longer to the shorter boundary ensures that the markers do not end up too far away from each other. The ECMM is considerably faster than the LSM and, according to its developers, also more stable. In both schemes, if several markers are close to each other then the resultant force can be large and this leads to large marker migration speed, which can cause numerical instabilities. Thus, both methods place upper bounds on the speed by limiting the forces generated by the virtual springs (LSM) or electrostatic fields (ECMM).

A series of packages, Quimp²¹ and its successors Quimp2²² and Quimp3²³, also use virtual boundary markers to describe the evolution of cell shape during amoeboid motility. The original version, Quimp, was designed to quantify the fluorescent intensity around cellular perimeter. It uses an active contour method to identify and place a chain of nodes around the cellular boundary, forming a closed polygon that serves as an approximation of the cell shape. Though nodes can be deleted or added as the morphology changes, approximately 150-175 nodes are used to describe *Dictyostelium* cells. These nodes are placed for each frame of a movie, but there is no means of connecting nodes from one image to the other. An updated package, Quimp2 fixes this by tracking the position of each node over time, enabling the quantification of local membrane displacement. It also identifies whether nodes are extending or retracting. To establish the polarity of the cell, the initial location of the uropod can be manually identified in the first frame. The software then automatically updates the location of the uropod to the most retractive node in the area of the uropod from the previous frame. The assumption is that the location of the uropod is relatively stable.

Alternatively, the uropod can be selected automatically as the most retractive node in each frame. Quimp3 adds the capability detecting and tracking pseudopods automatically. Pseudopods are identified as regions that are “outward extensions of a spherical cell.” Protrusions are defined as regions that satisfy minimal convexity, time and area changes over time. Growing pseudopods are protrusions with continuous positive area gain and where the cumulative gain is above a given threshold. Once identified, pseudopods are categorized based on their origin – do they split off from existing pseudopods or occur *de novo* – and fate – are they retracted or subsumed into the cell body (Fig. 4)?

Maeda and colleagues defined a circular map around the cell centroid and used the distance from the centroid to the cell membrane as the primary variable to analyze motion patterns of migrating *Dictyostelium* cells²⁴. They found ordered shape patterns by computing the autocorrelation function of this variable over space and time. They also defined a variable to describe molecular concentrations (using fluorescently-tagged markers) around the membrane using the circular map. The cross-correlation function between the distance from the centroid and the fluorescent intensity was used to determine how fluctuations in molecular concentrations influence local shape changes. The circular map has the advantage of simplicity. However, to construct a valid circular mapping, the cell shape cannot be overly convoluted. This criterion may not always be satisfied by chemotaxing cells with hyperactive pseudopodia.

Recently, Xiong and colleagues proposed a series of algorithms to detect and track pseudopodia during amoeboid motility using *skeletonization*. This is a technique from morphological image processing that reduces a shape into a series of connected lines – the skeleton – that roughly maintains the form of the shape (Fig. 5)²⁵. A moving cell gives rise to skeletons that change over time. Their evolution can be tracked relative to shape differences between consecutive frames to indicate the position and direction of these pseudopodial extensions and retractions. Because skeletonization uses topological and geometrical information from the whole shape and takes into account the relationship between the local curvature and the complete boundary curve, it is relatively more forgiving in terms of the spatial and temporal resolutions required during imaging. This is particularly advantageous when analyzing movement of fluorescent images, as it reduces phototoxicity. A possible drawback of the skeletonization technique is in tracking bleb-like shapes which are relatively small compared to stable protrusions, seen in some migratory cells^{26, 27}, because the local curvature around such a structure is not high enough to elicit a branch of the skeleton pointing at it. However, if the bleb’s curvature is large, the skeletonization technique is still able to detect it.

Rather than focusing on a predefined specific group of characteristics of cell shape changes over time, some recent studies instead identify morphological features of moving cells from individual frames using techniques from the field of machine learning and pattern recognition. As an example of this static spatial analysis, Bakal and colleagues²⁸ used segmented images to determine 145 morphological features in *Drosophila* BG-2 cells, imaged under one of 249 gene-overexpression or double-stranded RNA treatments. Seven treatment conditions that produced phenotypes qualitatively distinctive from control cells were selected as the features for training by a neural network. These were applied for each

cell in a specific treatment condition, and seven corresponding neural network Z-scores were computed. After averaging each Z-score over all cells in the same treatment condition, a 7-dimensional vector was used as a quantitative morphological signature of the treatment. The resultant 249 vectors were then classified into 41 different “phenoclusters” which revealed how local signaling networks regulated cell morphology. In another study, Keren and colleagues represented each cell boundary by a 400-dimensional vector representing the 2-dimensional Cartesian coordinates of 200 evenly-spaced points used to generate uniform cubic B-splines approximating the boundary of fan-shaped fish keratocytes²⁹. Applying principal component analysis to the population of cell shapes, distinct “shape modes” were found to describe variances in shapes. Among these, four primary shape modes were identified that account for 97% of the total variation of the shape. Geometric characteristics were then mapped to these modes, as well as coupled to molecular mechanisms involved in cell motility. These two studies represent a possible new trend of applying advanced data analysis techniques from computer science to cell shape analysis. They show great potential for helping to extract information from large data sets, which are easily acquired owing recent developments in imaging technology.

Dynamic shape evolution during migration

More than thirty years ago, in one of the earliest quantitative studies of motion in *Dictyostelium* cells, Potel and Mackay³⁰ showed that preaggregation cells perform a persistent random walk³¹ with an exponentially distributed memory of movement having a mean of approximately five minutes. This analysis was obtained by tracking a single spatial coordinate per cell per frame over time. Though crude by today’s standards, this represented a significant step in the description of cell migration. More recently, Li and Cox³² revisited these experiments and determined that *Dictyostelium* cells move forward in a zig-zag manner, making turns every 1-2 min on average, biasing their motion by remembering the last turn and turning away from it. Analyzing pseudopodial activity in preaggregation cells, Van Haastert and Bosgraaf determined that persistent motion arises because existing pseudopods split, whereas turns occur because pseudopods are formed in new locations³³. In this model of motility, the degree of persistence is determined by the ratio between splitting and *de novo* pseudopods: more *de novo* pseudopods lead to less persistent migration.

Unlike these reports, which analyzed unstimulated cells, most studies of *Dictyostelium* motility consider cells during their starvation-induced developmental stage, when cells chemotax in response to gradients of cAMP. A crucial question in understanding chemotaxis is how the chemoattractant gradient directs cellular shape changes. Analyzing the behavior of *Dictyostelium* cells in response to shallow cAMP gradients, Andrew and Insall showed that pseudopods are usually generated by splitting of existing pseudopods rather than by *de novo* formation³⁴. When *de novo* pseudopods do appear, their location and direction appear to be random, suggesting that cAMP does not regulate the formation of new pseudopods. The lifetime of a pseudopod is regulated by the chemoattractant gradient: when multiple pseudopods exist and compete to guide the direction of chemotaxis, the one that experiences the highest chemoattractant concentration wins out. Bosgraaf and Van Haastert confirmed these findings regarding the splitting and formation of *de novo* pseudopods for cells chemotaxing in shallow gradients³⁵. Furthermore, they found that when pseudopods split,

they do so at approximately 60 degree angles. Moreover, the splitting appears to alternate between left and right sides, but the size and frequency of pseudopods is independent of local cAMP concentration³⁶. This pattern is similar to that of randomly migrating cells but differs in that starvation inhibits the formation of *de novo* pseudopods. This strategy is different from that suggested for chemotaxing dendritic cells in which the probability that a lamellipod is extended locally is higher upon a localized increase in chemoreceptor occupancy³⁷. Skeletonization analysis revealed that 74% of pseudopods appear through splitting, which can be seen in Fig. 5c²⁵. The presence of many small, short-lived pseudopods was also observed. These may be missed by visual identification (because of their brevity) or by other techniques that require a minimum size for a membrane extension to be classified as a pseudopod. Owing to their limited lifetime, these short-lived pseudopods do not seem to contribute much to the overall cell displacement. Nevertheless, they may play a role in sensing or interpreting the gradient. Moreover, different strains were observed to generate varying numbers of these short-lived pseudopods, which may influence the cell's chemotactic efficiency.

It is natural to ask which signaling molecules control pseudopod formation and how these mechanisms are connected to the external chemoattractant gradient (Fig. 6). The traditional view of chemotaxis has been that chemoattractant receptor occupancy increases actin polymerization at the front of the cell through the PI3K pathway or other parallel pathways including PLC, PLA₂, and the TorC2 complex^{2, 9}. Andrew and Insall showed that the frequency of formation of pseudopods, but not their directionality, is regulated by PI3K signaling³⁴. This observation is consistent with measured increases in actin polymerization in cells lacking the PI3K antagonist, PTEN³⁸. Though *pten*- cells have difficulty chemotaxing, this is primarily because they have a hyperactive actin cytoskeleton, not because of an inability to interpret the external gradient. These studies also suggest that the pseudopod suppression is just as important as pseudopod formation in directing a cell. A cGMP-mediated signaling pathway that leads to myosin filament formation at the sides and rear of the cell has been shown to suppress lateral pseudopods^{25, 39, 40}. The increases in myosin-II concentration along the side can be seen as helping to maintain the cell's polarized shape. A viscoelastic model showed that retraction force is needed at the sides of the cell to maintain its shape⁴¹. Using traction cytometry, Meili *et al.* showed that these forces exist and depend on myosin-II⁴². The inhibition that prevents formation of lateral pseudopods may come from the local mechanical resistance provided by the cortex. Cells lacking dynacortin, an actin crosslinker, are approximately 30% less stiff than wild-type control cells⁴³. They send out multiple pseudopods that radiate at a broader distribution of angles relative to the chemoattractant gradient yet move with similar velocities as wild-type cells⁴⁴.

Discussion and conclusions

Because amoeboid motility involves continuous changes in cell shape, its proper analysis requires the separation of local deformations from cellular motion. Though traditional studies of motility have chiefly been concerned with the latter, we are now seeing a number of automatic image processing techniques that can be used to study cells based on these evolving morphologies. This new view on motility is revolutionizing our understanding of

chemotaxis in *Dictyostelium* and neutrophils by providing a quantitative way of coupling chemoattractant gradient sensing with directed cell motility⁹.

The different algorithms have advantages and disadvantages. Quimp is available as a plug-in for the widely used and freely available ImageJ (NIH) making it portable and accessible, particularly to experimental biologists. The skeletonization and LSM methods are implemented in MATLAB (The MathWorks, Natick, MA), a general purpose computational platform that is widely used by engineers and mathematicians, but is less commonly used by biologists. DIAS is a stand-alone commercial package. LSM and skeletonization work well with widely varying cellular shapes and so do not require high temporal resolution. In contrast, boundary markers and radial distances usually require less change in morphology and hence higher resolution. The LSM has the advantage over other techniques in that it is straightforward to incorporate viscoelastic models of cells that can then be used to simulate cellular motion⁴¹.

Shape analysis can be of interest in and of itself – for example, enabling researchers to detect subtle phenotypes. However, when the analysis is coupled to spatio-temporal changes in the underlying organization of the cytoskeletal network, as observed using fluorescence microscopy or other experimental techniques like traction cytometry⁴², it can lead to more thorough understanding of cellular migration. Several of the techniques described already include the facility to image fluorescent markers concurrently. Temporal and spatial correlation between shape features, such as pseudopods, and fluorescent intensities can shed light on the organization of the system^{21, 24, 25}.

Except for DIAS, the techniques described above used to analyze two-dimensional images of cells. Ultimately, the complete analysis of cellular morphology will require that we analyze cell motility in three dimensions. Two of the methods described above, LSM and skeletonization, have straightforward mathematical extensions to three-dimensional shape analysis, though the computational burden increases greatly. More likely, three-dimensional image acquisition may present a greater hurdle. When the imaging is carried out using fluorescence confocal microscopy, phototoxicity will be significant because of the long periods of light exposure that are needed to attain the vertical resolution required for reconstructing the shape accurately. Other imaging modes are possible – for example, the three-dimensional analysis carried out by DIAS is based on sections using DIC images – but analyzing fast moving cells requires high temporal resolution and this may require custom hardware and software¹⁷.

An open question is whether these techniques will be applicable to other cell types that have significantly different shapes. We foresee that the use of skeletons is particularly well suited to cells that have a number of extended protrusions and so would be adopted easily to analyze cells such as neuronal growth cones. On the other hand, when tracking cells whose boundary is considerably smoother, such as fish keratocytes, boundary marker tracking techniques (like those used in Quimp or ECMM) would be more appropriate.

The algorithms described here have been made possible by adapting tools from computer vision, including level set methods and deformable templates, to the description of cell

morphology. Despite these inroads, the computer vision literature is vast, and we foresee that it will continue to impact the analysis of cellular motility. For example, the algorithms discussed above do not use the temporal evolution of the image explicitly when identifying cells. The use of particle filters – a technique in which past observations are used to estimate the new contour⁴⁵ – could help to improve the tracking of cells. Similarly, generalizations of the principal component analysis⁴⁶ technique described above to movies could help identify modes of shape evolution. We envision that these and other techniques will further influence the study of cell motility.

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Fig. 1. Segmentation of digital images

a.b. Two successive frames of a chemotaxing *Dictyostelium* cell (the chemoattractant gradient is formed by a micropipette containing cAMP to the left of the images). The cell has been tagged with mCherry-dynacortin and GFP-myosin-II²⁵. These two images will be used to illustrate the different image processing algorithms. Time signatures are in seconds. The movie can be found at <http://www.biomedcentral.com/1752-0509/4/33/additional/>. c. Combined fluorescent intensity of GFP-myosin-II and mCherry-dynacortin for the image of panel a. Segmentation can be done by selecting pixels whose intensity is above a given threshold. d. Illustration of active contours on the combined fluorescent intensity for the image of panel b²². The cell is surrounded by a closed-curve. This is a manual initialization. The contour then closes in on the cell according to virtual forces generated by pixel intensities. e.f. Resultant segmented images superimposed over the images in panels a and b. g. The two segmented images superimposed. Blue and red correspond to the images in panels a and b, respectively.

Fig. 2. Level set method

Potential functions (top panels) are formed from the segmented images (the orange-colored bold lines in the bottom panels, which show different contours) according to the *signed-distance function*. This assigns a value whose magnitude is the distance from any pixel to the cell boundary and is positive or negative if on the inside or outside of the cell, respectively. The key to determining the temporal evolution of cellular morphology is to connect the two potential functions.

Fig. 3. The electrostatic contour migration method

In this scheme, the boundaries from two successive movie frames are superimposed. Points where the two boundaries overlap are stationary points (denoted by the circles). The edges between these stationary points denote the sectors. Within each sector, virtual electrostatic charges are imposed. The positive (“+”) charges are placed on whichever boundary is longer in each respective segment. The negative charges (“-”) go on the shorter. These charges give rise to electric field lines (grey lines) which cause positive charges to migrate.

Fig. 4. Cell tracking and automatic pseudopod detection using Quimp2

a. Superimposed images of the migrating cell. b.c. Single frames (those of Fig. 1). The lines show the location and lifetime of detected pseudopods. Panel c. shows a very long-lived pseudopod closely aligned with the gradient. The two pseudopods in panel b are much shorter-lived.

Fig. 5. Pseudopod detection through skeletonization

a.b. The green lines show the skeleton of the segmented images. Based on the evolution of these skeletons, pseudopods are marked in red (protrusions) or blue (retractions). c. This graph shows the temporal evolution of the different pseudopods. Red dots are protrusions, blue dots are retractions, and the green lines mark points that are connected. Splitting pseudopods are easy to detect in this plot. Note that the software can join two pseudopods activities more than two frames apart even if it is not seen in an intervening frame. This plot also shows the evolution of the long-lived pseudopod marked in Fig. 4c. It appears as the long interconnected series of red dots found roughly aligned with 0°.

Fig. 6. Pseudopod formation during chemotaxis

New pseudopods primarily appear because of splitting of existing ones. When two such pseudopods exist, the one experiencing the highest chemoattractant concentration tends to win out. Occasionally pseudopods can appear *de novo*, though these are suppressed by cGMP, myosin-II, dynacortin and other cross-linkers. PI3K contributes to the overall formation of pseudopods, but not their localization. Myosin-II forces on the side also help maintain the shape of polarized cells.