

Under-Agarose Folate Chemotaxis of *Dictyostelium discoideum* Amoebae in Permissive and Mechanically Inhibited Conditions

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ABSTRACT

Under-agarose chemotaxis has been used previously to assess the ability of neutrophils to respond to gradients of chemoattractant. We have adapted this assay to the chemotactic movement of Dictyostelium amoebae in response to folic acid. Troughs are used instead of wells to increase the area along which the cells can be visualized and to create a uniform front of moving cells. Imaging the transition zone where the cells first encounter the agarose, we find that the cells move perpendicular to the gradient and periodically manage to squeeze under the agarose and move up the gradient. As cells exit the troughs, their cross-sectional area increases as the cells become flattened. Three-dimensional reconstruction of confocal optical sections through GFP-labeled cells demonstrates that the increase in cross-sectional area is due to the flattening of the cells. Since the cells locally deform the agarose and become deformed by it, the concentration of the agarose, and therefore its stiffness, should affect the ability of the cells to migrate. Consistent with this hypothesis, cells in 0.5% agarose move faster and are less flat than cells under 2% agarose. Cells do not exit the troughs and move under 3% agarose at all. Therefore, this assay can be used to compare and quantify the ability of different cell types or mutant cell lines to move in a restrictive environment.

INTRODUCTION

Dictyostelium discoideum is a eukaryotic amoeba that normally inhabits the soil. During its life cycle, the haploid cells undergo two distinct types of chemotactic movement. In the vegetative phase, the amoebae are attracted to folic acid, which is released by their bacterial food source, and detected by cell surface folate receptors (6,13). As the bacterial food source is depleted, *D. discoideum* enters the developmental stage of its life cycle. The number of folate receptors decreases during the first 7–9 h of development (11), and the cells become responsive to cAMP released by other amoebae (8,13). The number of cAMP receptors (cARs) begins to increase immediately after the initiation of starvation, and cAR1 is maximally expressed on the surface 3–4 h into development (7).

Cell motility is most often studied in a laboratory by observing cells crawling on a glass cover slip in liquid medium. Under these conditions, there is little to resist the movement of the cells except their own adhesion to the substrate. However, cells in natural environments, such as amoebae moving in the soil or neutrophils extravasating through the endothelium of a capillary, are presumed to move under more restrictive conditions. In addition, movement in 3-D environments has the added complexity that cells do not have a clearly defined dorsal and ventral surface because they can interact with the substrate on all sides. The molecular mechanisms underlying motility in 3-D environments are as yet poorly defined.

We sought to develop an assay in which cells attempt to move in a restrictive environment, but where high-resolution imaging is possible. Under-agarose assays have been used to show the chemotactic and invasive abilities of neutrophils and macrophages (2,12). *Dictyostelium* cells had previously been shown to be able to move under agarose by Yumura and co-workers (18) as a way to image moving cells while limiting their three-dimensionality. Here, we have modified the under-agarose chemotaxis assay by altering the well configurations and varying the agarose concentration to investigate the chemotactic motility of *Dictyostelium* cells in different types of restrictive environments. Using time-lapse imaging and confocal microscopy, we show that cells deform the agarose but at the same time become flattened as they move under the agarose. The assay is reproducible and movement is quantifiable, allowing detailed analysis of the motility of individual cells or populations of cells.

MATERIALS AND METHODS

Cell Culture

NC4A2 cells were transfected with vectors that express cytosolic GFP (pB15-GFP) (15), GFP-ABD120 (14) to visualize F-actin, and myosin II heavy chain-GFP (mhcA=GFP) (10) to visualize myosin II localization. All cells were cultured in HL-5 medium [5 g Bacto® Proteose Peptone no. 2 (Difco, Detroit, MI, USA), 5 g BBL Thiotone E, 10 g glucose, 5 g yeast ex-

tract, 0.35 g Na_2HPO_4 , 0.35 g KH_2PO_4 , and double-distilled water to 1 L, pH 6.7].

Under-Agarose Chemotaxis Assay

To prepare plates for the under-agarose assay, SeaKem® GTG agarose (BMA, Rockland, ME, USA) was melted at concentrations as indicated in SM medium [10 g Bacto-Peptone (Difco), 10 g glucose, 1 g yeast extract, 1.9 g

KH_2PO_4 , 0.6 g K_2HPO_4 , and 0.43 g MgSO_4 to 1 L, pH 6.5]. Similar results were obtained with other types of agarose [ultraPURE™ LMP (Invitrogen, Carlsbad, CA, USA), NuSieve® GTG (BMA), and Ultra Low Gelling (Fisher-Biotech)]. Motility was generally higher when SM was used to prepare the agarose instead of HL-5. The agarose mixture was prepared fresh each day by mixing sterile SM (previously autoclaved) with the agarose powder and au-

toclaving for 5 min, slow exhausting, and then plating as soon as possible. Agarose solution (4 mL) was added to each 60-mm plastic petri dish and allowed to harden for 1 h. Three troughs were then cut in the agarose using a single-edge razor blade. The troughs were 2 mm wide, the length of the blade 39 mm long, and spaced 5 mm apart (Figure 1).

The agarose was removed to form the troughs using light suction from a Pasteur pipet applied to the "end" of the agarose plug to be removed. Care was taken at each step not to disturb the agarose interface at the edge of the trough. A solution of 100 mM folic acid (Research Organics, Cleveland, OH, USA) was prepared by dissolving 0.44 g folic acid in approximately 200 μL 10 M NaOH. This was brought to a final volume of 10 mL, adjusted to pH 7.0, and stored in the dark at 4°C. A 0.1-mM folic acid solution was prepared from the stock solution, and then 40 μL were added to the center trough (Figure 1) and the gradient was allowed to develop for 1 h before adding cells.

While the gradient developed, cells were prepared for addition to the troughs. It was found that cells were reduced in their chemotactic response if grown to stationary phase or if centrifuged to concentrate and wash the cells. To maximize responsiveness, cells were plated at 4×10^5 cells/mL in 10 mL HL-5 in 100-mm petri dishes and grown for 2 days to a titer of approximately 1×10^6 /mL. The overlying media was aspirated, and the cells were resuspended in fresh HL5. For individual cell analysis, the cells were resuspended in 10 mL HL5, counted with a hemocytometer, and the density was adjusted to 1×10^6 /mL. For population analysis, the cells were harvested in 1 mL HL5 and adjusted to 1×10^7 cells/mL. Seventy microliters of cells were then added to the peripheral troughs.

For fluorescence imaging experiments, 0.75 mL agarose was added to a Rose chamber (21) or a 60-mm glass bottom petri dish (Willco Wells, Amsterdam, The Netherlands) so that cells could be imaged through a 0.17 mm thick glass cover slip. The troughs in the Rose chamber were cut with a shortened razor blade to a length of 10 mm, and the amount of cells and folate was decreased proportionally.

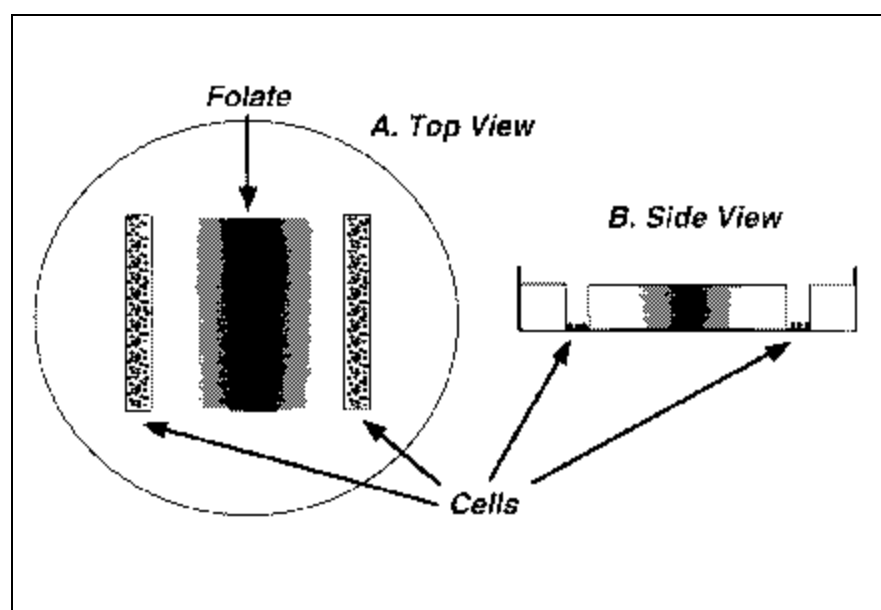


Figure 1. Experimental setup. (A) The under-agarose assay plate. A 60-mm petri dish is filled with agarose, and three troughs are then cut with a razor blade. The center trough is filled with chemoattractant, and 1 h later cells are added to the outside troughs. (B) Side view of the plate. Cells can only move out of the troughs by squeezing between the deformable agarose and the non-deformable plastic or glass surface.

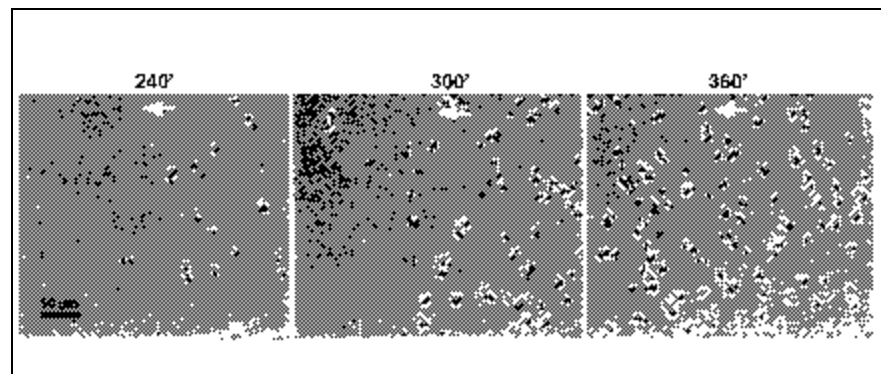


Figure 2. Under-agarose chemotaxis of Dictyostelium cells to folic acid. Images were taken of cells at three times during movement under 0.5% agarose toward the folate source. Chemotaxis to 0.1 mM folate was imaged under 0.5% agarose. The images were taken at a fixed location about 1500 μm from the edge of the cell trough, showing the movement of the front of cells across the plate. The 240' time point is equivalent to 4 h after the addition of cells to the trough. Arrows indicate the origin of gradient and the direction of cell movement. A QuickTime movie showing the chemotactic movement of cells can be accessed at <http://www.BioTechniques.com/Movies/Nov01/KnechtFig2.mov>.

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Analysis of Chemotaxis

Images were taken of the cell populations using a Zeiss® inverted microscope (Carl Zeiss, Oberkochen, Germany), Paultek Imaging CCD camera (Advanced Imaging Concepts, Princeton, NJ, USA), Scion LG3 frame grabber (MVI, Avon, MA, USA), and NIH Image software (NIH, Bethesda, MD, USA). Cell population speed was determined by measuring the distance the leading cells had moved away from the trough edge at a particular time point. Data from 10 cells were used to calculate the average distance the front-most cells had migrated. The distance moved was determined by imaging a stage micrometer and superimposing it on the images. The time it took the cell front to travel to successive time points was used to determine cell front speed. Individual cell speed and direction change were determined using DIAS® software (Solltech, Oakdale, IA, USA). Speed was calculated from the displacement of the centroid from frame to frame. Direction change was measured as the absolute value of the difference in the direction of movement of the centroid from frame to frame, measured in degrees. Cross-sectional area measurements were made using NIH Image software. The cross-sectional area is measured as the area of the image of a cell seen using phase microscopy. Confocal imaging of GFP-labeled cells was performed using an MRC 600 (Bio-Rad Laboratories, Hercules, CA, USA) equipped with a 25-mW krypton-argon laser and COMOS software. Three-dimensional reconstruction was done using Viewpoint3 and Volrend software written by Dr. Eric Shelden (University of Michigan). Links to QuickTime™ movies are shown in the legends to Figures 2, 3, and 6.

RESULTS AND DISCUSSION

Development of the Under-Agarose Assay

The traditional under-agarose chemotaxis assay of macrophages and polymorphonuclear leukocytes (PMNs) utilizes small round wells cut in agarose into which cells or chemoattractant are

loaded (2,12). This format provides a small area where the cells are able to move in the direction of the gradient, making imaging of the transition zone difficult. We have modified this assay to make it more suitable to the visualiza-

tion of a population of moving cells, allowing for quantification of both population and individual cell behavior. By cutting linear troughs instead of circular wells, movement along the entire front of the trough could be visualized and

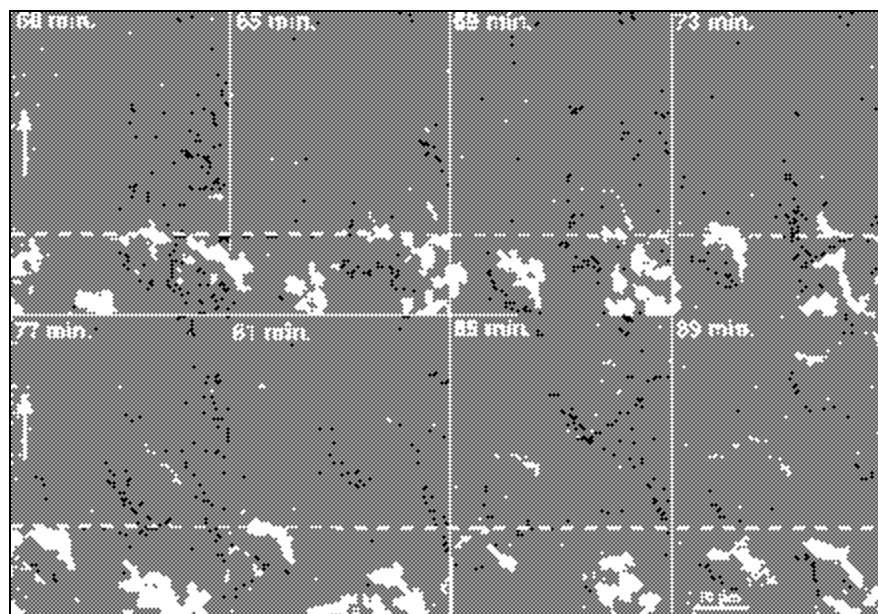


Figure 3. Movement of cells at the transition zone. The large arrow points toward the source of folate and the direction of cell movement. The dashed line indicates the edge of the cell trough. Cells above the dashed line have moved underneath the 2% agarose sheet. Note the increased cross-sectional surface area of the cells. The time points indicated are time elapsed after application of cells to trough. The black arrowhead indicates a cell that has moved under the agarose during this sequence. A QuickTime movie of cells exiting the trough can be accessed at <http://www.BioTechniques.com/Movies/Nov01/KnechtFig3.mov>.

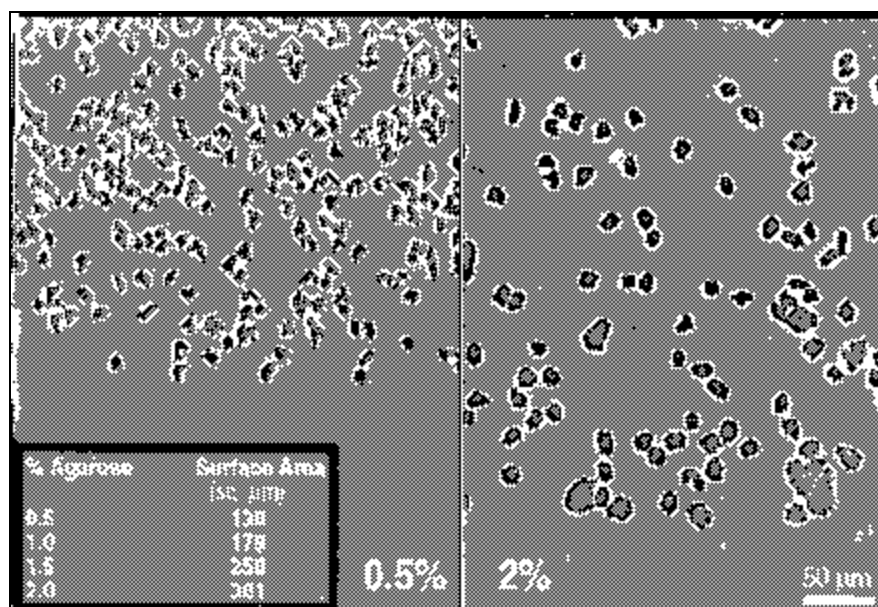


Figure 4. The cross-sectional area of cells under agarose is increased. Images of cells under 0.5% and 2% agarose at the leading front, 1500 μ m from trough edge. The arrow indicates the direction of movement. The inset shows the quantification of the cross-sectional area of the cells for the indicated agarose concentrations. Note the increased area of the cells under 2% agarose.

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quantified. In preliminary experiments, it became clear that, if care was not taken in the cutting of the troughs, the interface could be disturbed such that gaps were created that allowed cells under the agarose. Even when care was taken, a few small areas where cells exited the trough much more rapidly were sometimes observed. These areas were ignored in analysis, as they represented a small proportion of the behavior along the entire trough front. When the agarose was lifted and rotated within the petri dish to purposely disturb the interface, cell migration was less inhibited at all agarose concentrations. We believe this indicates an interaction between the agarose and the plastic substrate that must normally be broken or disturbed by the cells moving out of the trough.

Using this assay, *Dictyostelium* cells were visualized over time after placement in the trough in the presence of a folate gradient. Cells exited the trough and moved up the folate gradient for at least 10 h (Figure 2). In the first hour after application of cells to the trough, approximately 10 times as many cells moved out of the trough toward the chemoattractant than away (data not shown). After 4 h, the differential in movement approaches 1000-fold. When cells were assayed using plates in which no folate was added, the results were similar to the results from the distal side of the chemotaxis assay. Thus, randomly moving cells do not significantly exit the troughs, and it is the attraction of the folate gradient that leads to cells moving under the agarose.

Transition and Cortical Deformation

Chemotaxis was initially characterized using troughs cut in either 0.5% agarose (the minimum mechanically workable concentration) or 2% agarose. Before the arrival of cells, the agarose is in direct contact with the plastic surface of the petri dish. At the trough edge interface, the cells might move by climbing up and over the agarose, traveling through the agarose, moving under the agarose, or stopping at the edge of the trough. Microscopic analysis confirms that the cells do not climb over or through the agarose. Many but not all cells move out of the trough and under

the agarose in the time frame of the assay. Cells were not immediately able to make the transition to movement under the agarose when they reached the edge of the trough. Time-lapse movies of the trough edge show cells moving back and forth along the trough edge, and only occasionally does a cell actually extend a pseudopod under the agarose and move up the gradient. This behavior is particularly evident in the 2% agarose plates. There is a continuous exit of cells from the trough throughout the assay. Some cells are left behind and do not exit the trough regardless of the concentration of the agarose. These cells are primarily in the middle or distal portion of the trough. This cellular behavior suggests that a stable gradient does not extend very far into the trough.

For cells to move out of the trough, they have to flatten themselves to a thickness that will fit between the agarose and the plastic, deform the agarose upwards while maintaining shape, or a combination of the two such that they deform the agarose upwards as they flatten themselves. As cells leave the trough and move under the agarose, there was a dramatic increase in their 2-D cross-sectional area, indicating a flat-

tening of the cells (Figure 3). The average cross-sectional area of cells in the trough was $32 \pm 4 \mu\text{m}^2$, while cells 1500 μm from the trough edge had an average area of $130 \pm 4 \mu\text{m}^2$ under 0.5% agarose. If the stiffness of the agarose were causing the flattening of the cells, then, as the concentration of agarose is increased, the cells should become flatter. Consistent with this hypothesis, cells under 2% agarose concentration had a cross-sectional area of $301 \pm 25 \mu\text{m}^2$ (Figure 4 and inset).

Another approach used to assess changes in cell morphology was to use confocal imaging to determine the 3-D shape of the cells under agarose. Cytosolic GFP was expressed in transfected *Dictyostelium* cells. The chemotaxis assay was set up in a Rose chamber on a cover slip to allow high-resolution 3-D optical sectioning with the confocal microscope. Reconstruction (Figure 5) of the optical sections showed a clear flattening of the cells under agarose as compared to cells remaining in the trough. Cells in the trough that had not yet moved under the agarose were variable in height, ranging as high as 12 μm . Cells that had crawled under the 0.5% agarose were 8–10 μm thick, and

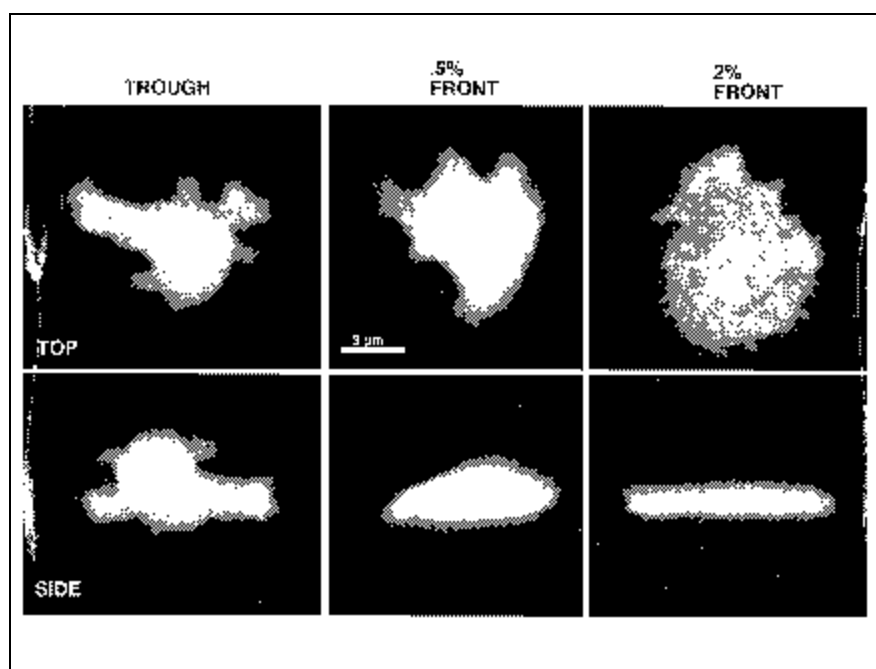


Figure 5. Volume reconstruction of cells. Cells expressing cytosolic GFP were imaged in the trough, under 0.5% or 2% agarose. “Front” cells images were captured 1500 μm from trough edge. Confocal optical sections were collected through each cell and then reconstructed into a 3-D volume. Each volume is shown in a top down and side view.

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cells under 2% agarose were uniformly 4 μm thick.

Another approach to understanding the interaction of the cells with the agarose is to determine the extent to which the agarose is deformed upward by the cells moving underneath. Since the cells have a thickness of 4 μm under the 2% agarose, it is likely that the agarose is locally deformed or moved upward as the cell passes underneath. To visualize this deformation, 0.5 μm fluorescent beads were added to the agarose before hardening. Confocal images with a Z-section approximately 1 μm thick were collected with the focal plane fixed 4–5 μm above the substrate, near the top of the cells. Beads could clearly be seen to move into and out of the plane of focus as the cells moved underneath (Figure 6). Some beads (Figure 6, lower left) are visible before the cell arrives and then move out of the plane of focus as the cell passes. Beads at the bottom (Figure 6, upper right) of the agarose were frequently pulled out of the agarose and phagocytosed by the cells. The beads that remained in the agarose after the cells passed were usually in a slightly higher focal plane, indicating that the agarose is lifted slightly by the passing of cells. Thus, cells trailing the front will be exposed to a slightly different environment. If the imaging plane was set higher in the agarose, then no movement of the beads was observed. This indicates that the entire sheet of agarose is not raised but rather that the agarose is locally deformed above the cell. In addition, the beads did not move until just before (approximately 2 μm) the advancing cell reached it and returned to their positions just after the cell had passed, indicating that the deformation of the agarose is quite local.

Effect of Agarose Concentration on Cell Movement

After moving out of the trough, the cells form a distinct front that progresses toward the chemoattractant well (Figure 4). Time-lapse imaging and computer analysis were used to visualize and quantify the movement of cells up the folate gradient. The speed of the front was calculated by determining the position of the leading cells over time. Under

0.5% agarose, the cell front moved at 6.4 $\mu\text{m}/\text{min}$ (Figure 7 and inset). The rate of movement of the cells was surprisingly consistent throughout the assay.

Since the cells deform the agarose away from the surface to move, we hypothesized that, by increasing the concentration of the agarose, the cells would have to work harder to move.

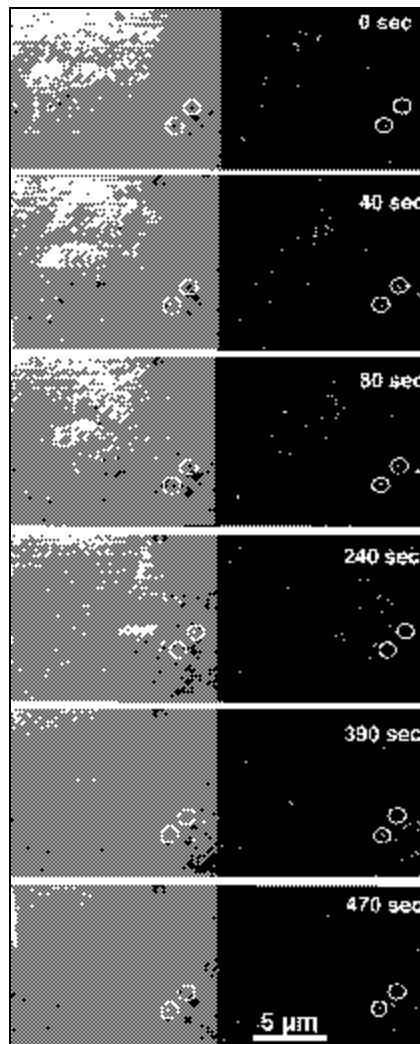


Figure 6. Agarose deformation assay using fluorescent beads. Fluorescent beads (FITC, 0.5 μm diameter) were added to 2% agarose before pouring the plates. Images were collected using the confocal microscope as cells moved through the field of view. The plane of focus was just above the height of the cells so that some beads are initially below the plane of focus and some are in the plane. As the cell moves by, beads below the plane are moved through the plane and so become temporarily visible. Beads initially in the plane move out of the plane, becoming invisible, and then reappear after the cell passes by. A QuickTime movie showing the movement of the beads can be accessed at <http://www.BioTechniques.com/Movies/Nov01/KnechtFig6.mov>.

Consistent with this idea, the speed of the front decreased as the agarose concentration increased (Figure 7 and inset). At 2% agarose, the cells moved at 3.1 $\mu\text{m}/\text{min}$; however, at 3% agarose, the cells can no longer move out of the trough, indicating that the cells are unable to deform this stiff matrix sufficiently to squeeze underneath.

These data may provide a clue as to why many previous attempts to use under-agarose chemotaxis with mammalian cell lines have been unsuccessful. Cells clearly undergo a flattening as they begin to protrude under the agarose. Even *Dictyostelium* amoebae that move robustly once under the agarose have a difficult time making the transition to flatten out and squeeze under the agarose (Figure 3). The edge of the agarose trough seems to present a restrictive barrier that cells must overcome to move up the chemoattractant gradient. Cells must have sufficient adhesion to the surface to apply a forward force and deform the agarose as they move, and they must have sufficient cortical tension not to be crushed by the downward force of the overlying matrix. Many cell types may be unable to meet both of these criteria to move under agarose. By varying the substrate composition and reducing the stiffness of the overlay, it may be possible to improve the movement of mammalian cell lines. In fact, Mollison et al. (9) have shown that varying the concentration of agarose affects the under agarose migration distance of PMNs.

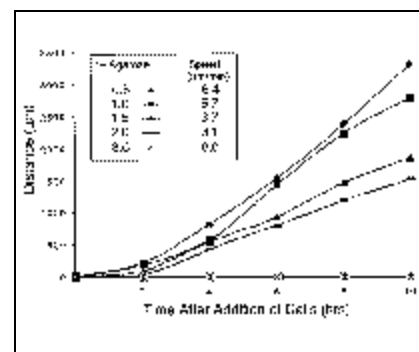


Figure 7. Movement of the leading edge of cells under various agarose concentrations. NC4A2 cells applied to troughs at the 0 time point. The movement of the front of cells over time is plotted. The cells at the front move at a constant speed throughout the assay. The inset shows the average speed of the front calculated from the slope of the 2–10 h time points.

Characterization of Chemotactic and Chemokinetic Motility

Cell motility was also analyzed by computerized motion analysis of individual cell paths over time. Cells at the leading front of 0.5% agarose had a mean velocity of $6.9 \pm 0.1 \mu\text{m}/\text{min}$ and

a very low rate of directional change, indicating that they were moving persistently up the folate gradient (Figure 8 and inset). This is also evident from the paths of individual cells, which are quite linear. The fact that the cell speed and the population speed are similar also indicates that cells are tracking

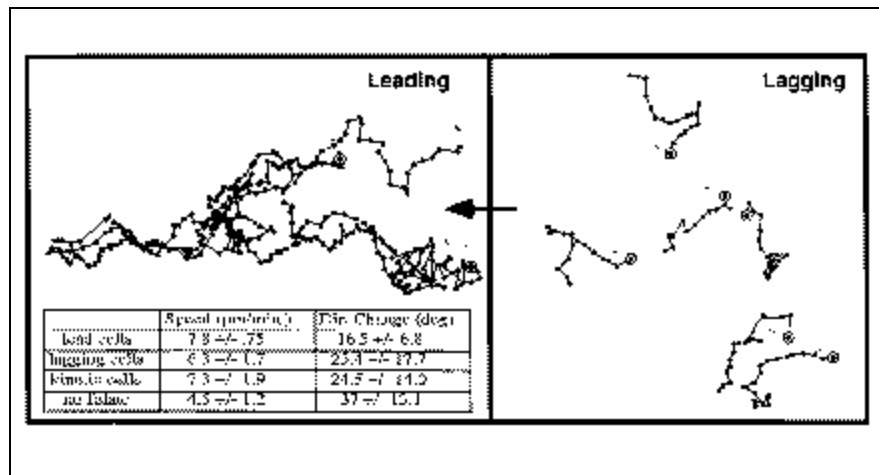


Figure 8. DIAS analysis of leading-edge and lagging cells. The paths of representative cells were measured using DIAS motion analysis software. The arrow points toward the chemoattractant trough and the direction of cell movement. Lagging cells are approximately $450 \mu\text{m}$ behind the leading cells. The inset table shows the quantification of speed and direction of leading and lagging cells as well as cells exposed to no folate or a uniform concentration of folate throughout the agarose. The speed and direction changes are expressed as the mean value of at least 50 cells monitored for at least 30 min in two independent experiments (mean \pm SEM).

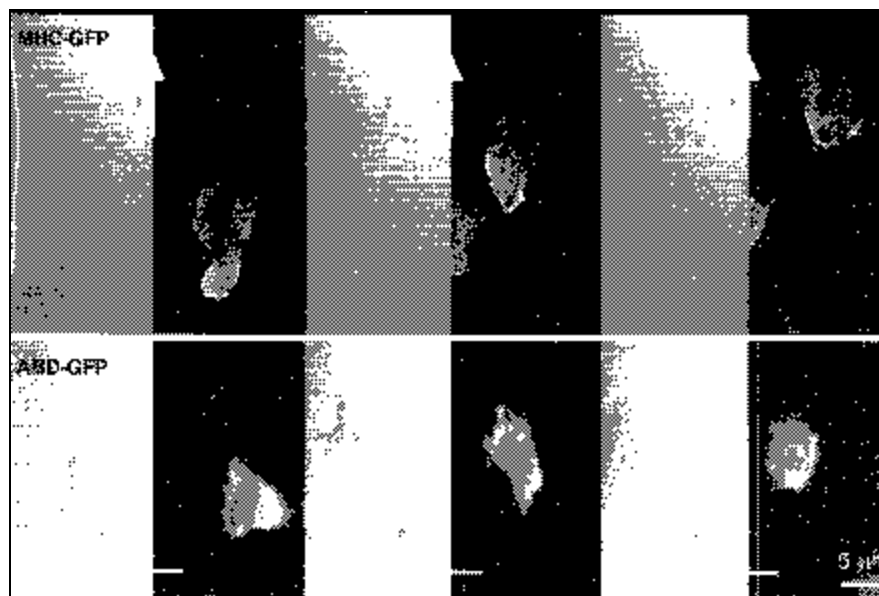


Figure 9. GFP-labeled cells chemotaxing under agarose. ABD120 and myosin II heavy chain-GFP (mhcA-GFP) fusion proteins were expressed in cells to visualize F-actin and myosin II in cells. The top panel shows a mhcA-GFP-transformed cell, and the bottom panel shows a GFP-ABD120-transformed cell. Cells are imaged under 2% agarose using confocal microscopy as described. Cells are moving in the direction of the arrows.

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consistently up the gradient. The cells behind the leading front were slower ($5.0 \pm 0.7 \mu\text{m}/\text{min}$) but had twice the rate of directional change. The more frequent turns are evident in the paths of the trailing cells (Figure 8). This behavior is most likely due to the disturbance of the gradient by the leading cells. Dictyostelium cells have both cell surface and secreted folate deaminases that will alter the local gradient as the leading cell passes.

Chemotactic agents can have both chemotactic (directional) and/or chemokinetic (speed) effects on cells. To determine the effects of folate on *D. discoideum* cells, we compared the movement of cells in the standard chemotactic chamber to movements in a chamber in which the folate was present uniformly throughout the agarose and the troughs, or a chamber that contained no folate. Cells in a uniform con-

centration of folate moved as fast as cells exposed to a gradient (Figure 8 and inset), but the cells made 50% more direction changes. There was also a 50% decrease (data not shown) in the number of cells migrating out of the troughs compared to the chemotactic conditions. Unlike the chemotactic assay, in the chemokinetic assay an equal number of cells moved out in both directions. When no folate was present, very few cells moved out of the troughs (data not shown). Thus, folate exerts both chemotactic and chemokinetic influences on cells. This is to our knowledge the first demonstration of a chemokinetic effect of folate. The evidence for chemokinesis is not definitive because the ability of Dictyostelium to bind folate and the presence of extracellular and cell-surface folate deaminases (3,4) may convert a uniform folate concentration into a local gradient

(1). It may be informative to perform this experiment using a non-hydrolyzable folate analog such as methotrexate to confirm this observation.

Localization of GFP-Labeled Proteins

One of the benefits of this assay is the ability to image cells that are actively undergoing chemotaxis in a mechanically inhibited environment. Confocal microscopy of cells expressing GFP-fusion proteins was used to examine the localization of several cytoskeletal proteins during chemotaxis. It has been shown previously that myosin II moves to the rear of the cell when cells are overlaid with a thin agarose sheet (2). The same phenomenon was observed when cells labeled with GFP-myosin II moved from the trough out under the agarose (Figure 9). The myosin II that

was previously throughout the cortex, rapidly relocalized to the rear of the cell. The F-actin cytoskeleton was also visualized using an actin binding domain-GFP fusion (18). Surprisingly, the bulk of the F-actin co-localized with myosin II at the rear of the cell rather than in the leading lamella (Figure 9).

The under-agarose chemotaxis assay has numerous potential applications that we will explore in the future. The assay has been combined with an electric cell impedance sensing (ECIS) system to allow the arrival of the cells at a specified location to be recorded without visualization (see following paper). This assay opens the way for a high-throughput screen for agonists and antagonists of chemotaxis. Also, a variety of *Dictyostelium* mutants are available with alterations in cytoskeletal and motility-related genes. It is likely that mutations that affect cortical integrity will have adverse effects on motility in this restrictive environment. Preliminary examination of cells lacking myosin II heavy chain indicates significant defects in movement under agarose (Laevsky unpublished observations and references. Furthermore, the visualization of GFP-labeled proteins in directionally moving cells will allow us to assess the localization of proteins in relation to the motility process and how the machinery changes between mechanically inhibited and uninhibited conditions. Last, it should be possible to adapt this assay to isolate the cells left in the trough as a way to screen for mutants deficient in force production or chemotaxis.

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