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Assaying Chemotaxis of *Dictyostelium* Cells

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Summary

Both prokaryote and eukaryote cells can sense and move up chemical concentration gradients (chemotax). As a means of finding food sources during vegetative growth, *Dictyostelium discoideum* naturally chemotaxes toward chemicals released by bacteria. As part of its developmental life cycle, *D. discoideum* chemotaxes towards cAMP. This chapter describes protocols for using *Dictyostelium* to understand the cell biology behind and the signaling events necessary for eukaryotic amoeboid chemotaxis. The chapter includes analyses of random cell motility, directed motility up chemical gradients, cellular responses to uniform chemoattractant exposure, and the utility of fluorescent probes for chemotaxis signaling events. Random cell motility in the absence of chemoattractant is analyzed to decipher the properties of self-organizing pseudopodia extension and retraction. Monitoring chemotaxis toward cAMP and folate allows the determination of signaling events required for sensing a chemical gradient and moving in a directed, persistent manner up the gradient. Uniform chemoattractant exposure is employed to elucidate the immediate intracellular responses to chemoattractant stimulation. Finally, analyzing cells expressing fluorescent fusion proteins is vital to elucidating the location of signaling events during chemotaxis.

Key Words: cAMP; amoeba; chemotaxis; *Dictyostelium*; folate; fluorescent probes.

1. Introduction

Dictyostelium discoideum chemotaxes by F-actin-mediated pseudopodia protrusion and myosin-II-mediated posterior retraction. This type of movement resembles the amoeboid chemotaxis undergone by human lymphocytes (reviewed in **refs. *1*,*2***). Thus, researchers have exploited *Dictyostelium's* genetic manipulability and developed the organism into a model system for studying amoeboid movement up chemical gradients.

Vegetative *Dictyostelium* cells randomly protrude pseudopodia in the absence of chemoattractant and in the absence of key signaling proteins required for

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chemotaxis (reviewed in **ref. *3***). Analyzing strains undergoing random motility will determine whether they are defective in the core signaling components required for pseudopod extension and retraction. In this assay, vegetatively growing cells are washed and seeded onto a chambered coverslip in starvation buffer. Cell movement is then recorded by acquiring a series of phase-contrast or differential interference contrast (DIC) images.

Dictyostelium chemotaxis toward cAMP under starvation conditions is widely used to assess chemotaxis competence. Analyses of chemotaxis up cAMP gradients were fundamental to discovering the essential role of phosphatidylinositol-3 kinase (PI3K), its downstream effecter protein kinase B (PKB), and the antagonistically acting phosphatase and tensin homolog (PTEN) phosphatase in sensing chemoattractant gradients and directing cellular movement up such gradients ***(4–8)***. Starvation of *Dictyostelium* induces a developmental program in which the cells secrete oscillatory pulses of cAMP waves every 6 min. These cAMP waves activate receptors at the cell surface and lead to upregulation of signaling proteins involved in sensing and chemotactically responding to extracellular cAMP. The waves also create a cAMP gradient and induce the cells to move toward the areas of highest cAMP concentration. After approx 9 h of starvation, the chemotactic aggregation of up to 105 cells leads to the formation of multicellular mounds (reviewed in **refs. *9*,*10***). For chemotaxis analysis, *Dictyostelium* is put into a starvation buffer and exposed to cAMP pulses for 5 h to mimic the intrinsic oscillatory pulses of cAMP that occur when *Dictyostelium* undergoes normal differentiation on a substratum. cAMP pulsing causes the cells to induce expression of early genes, including the cAMP receptor and other signaling proteins necessary for chemotaxis, which are normally expressed after 5–6 h of development ***(11)***. This chapter describes two common methods for developing *Dictyostelium* in preparation for chemotaxis to cAMP. The cells are then exposed to a cAMP gradient emitted from a micropipet and their chemotaxis recorded by acquiring phase-contrast or DIC images.

Problematically, some mutants exhibit defects in chemotaxis to cAMP after 5 h of pulsing, but not after 7 h of pulsing. Such discrepancies are widely attributed to *Dictyostelium* cell polarity being gradually strengthened as the cells become further developed. When highly polarized cells are exposed to a change in the orientation of the cAMP gradient, they U-turn into the newly positioned gradient. Less developed, and therefore less polarized, cells disassemble their old leading edges and reassemble new pseudopodia in the direction of the new gradient. This complication, in which the developmental program sometimes affects the chemotaxis phenotype, can be overcome by analyzing vegetative cell chemotaxis toward folic acid. During vegetative growth, *D. discoideum* chemotaxes toward folate and other nutrients released

by bacteria ***(9,10)***. Thus, folic acid can be used as a chemoattractant in much the same way as cAMP is used to create a chemoattractant gradient or uniform chemoattractant concentration ***(12)***. In this assay, vegetative cells are analyzed in starvation buffer containing 20% growth medium, which reduces the amount of ungraded folic acid in the milieu without inducing the developmental program.

For rigorous comparison of chemotaxis phenotypes, the cell polarity, directionality, and speed of centroid movement during chemotaxis should be quantitated. Computer-assisted analysis of cell movement and shape change is commonly performed using image analysis software, such as dynamic image analysis system (DIAS) software (Solltech, Oakdale, IA) ***(13)***, as described in Chapter 16. Generally, the perimeter (shape) and centroid position for 4–10 cells in frames 100–200 of a 300-frame movie are marked. The speed of centroid movement and changes in cell shape between different frames are calculated and averaged for a single amoeba moving for 10 min.

Analyzing cellular responses to uniform chemoattractant concentration ("global stimulation") allows the determination of whether a strain is able to sense and adapt to chemoattractant. In response to uniform chemoattractant exposure (<2% difference in concentration across the length of the cell), Ras, PI3K, and pleckstrin homology (PH) domain-containing proteins immediately translocate from the cytoplasm to the cell membrane (2–10 s poststimulation). The cells then adapt to the new chemoattractant level, causing PI3K-signalling proteins to be released from and PTEN to associate with the entire membrane (20–40 s poststimulation) ***(4–8,14,15)***. After adaptation, PI3K and its effectors re-associate with the membrane in patches that are the sites of pseudopodia extension and random motility behavior. Fluorescently labeled signaling molecules are key to monitoring protein localization and activity in global stimulation analyses as described later.

Fluorescent probes can be used in combination with any of the chemotaxis assays mentioned previously to assess protein localization and activation during random motility, chemotaxis, and global stimulation. In these experiments, proteins of interest are made as fusions with fluorescent proteins, such as green fluorescent protein (GFP), yellow fluorescent protein (YFP), cyan fluorescent protein (CFP), or red fluorescent protein (RFP). The fusion proteins are then expressed in *Dictyostelium* and used to determine the localization of the protein of interest during chemotaxis in vivo. GFP fusions of PI3K and PTEN have been critical in determining that these proteins translocate to the leading edge and posterior of chemotaxing cells, respectively (*see* **Fig. 1**) ***(4,5,7)***. The localization of fluorescent fusion proteins should always be confirmed by other methods, such as indirect immunofluorescence with antibodies to endogenous epitopes, to ensure that the large fluorescent protein tag does not cause an artificial localization. Further, as *Dictyostelium* is an experimental system in which gene knock-

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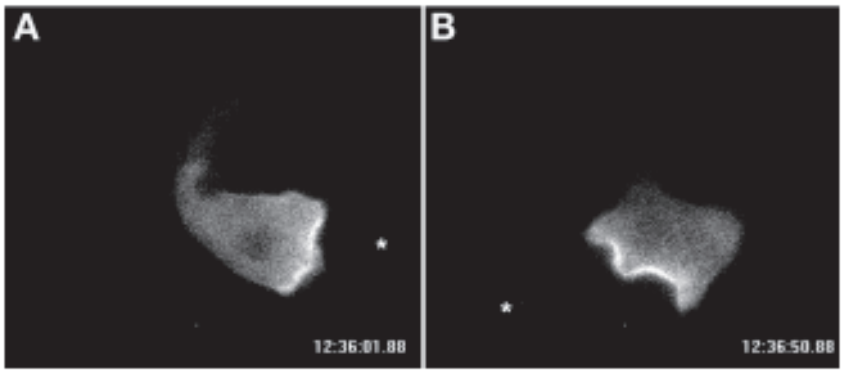

Fig. 1. Monitoring protein localization and activation using green fluorescent protein (GFP) fusion proteins. **(A)** A femtotip containing cAMP is placed on the right side of a *Dictyostelium* cell expressing GFP-PI3K. As the cell sensing the cAMP gradient moves towards the needle source (white asterisk), GFP-PI3K localizes to the leading edge. **(B)** If the needle is moved to the lower left side of the cell, (white asterisk), the *Dictyostelium* re-orients and creates a new leading edge with GFP-PI3K at the new front.

outs and gene replacements can be readily made, it should be demonstrated that the fluorescent fusion protein complements the null mutation or does not cause an abnormal phenotype when used to replace the endogenous copy of the gene.

Fluorescent protein fusions with protein domains are also used to assess the occurrence and location of protein activation in vivo. GFP fusions of the PH domains of PKB or cytosolic regulator of adenylyl cyclase (CRAC), which bind phosphatidylinositol 3,4,5 triphosphate (PIP3), are used to assess PIP3 accumulation and, therefore, PI3K and PTEN activity ***(5–8,16)***. A GFP fusion of the Ras binding domain (RBD) of human Raf1 has been used to detect biologically active, GTP-loaded Ras ***(15)***. The fluorescence intensity across the cells is quantitated using either ImageJ (National Institutes of Health [NIH], Bethesda, MD), Metamorph (Molecular Devices Corporation, Downingtown, PA), or IPLab-Spectrum (Scanalytics, Fairfax, VA) software. Simultaneous use of GFP and RFP fusion proteins will allow the more precise detection of the order of translocation and activation events (Sasaki and Firtel, unpublished data).

2. Materials

2.1. Buffers and Equipment

1. Na/K phosphate starvation buffer—one of two types.
   a. Na/K phosphate starvation buffer A: 12 m*M* Na/K phosphate buffer: 2.5 m*M* $Na_2HPO_4 \cdot H_2O$, 9.5 m*M* KH_2PO_4, pH 6.1. Autoclave to sterilize. Store at 22°C. Stable for 1 yr.

b. Na/K phosphate starvation buffer B: 10 m*M* Na/K phosphate buffer: 5 m*M* Na_2HPO_4, 5 m*M* NaH_2PO_4, 2 m*M* $MgSO_4$, 200 µ*M* $CaCl_2$, pH 6.2. Autoclave to sterilize. Store at 22°C. Stable for 1 yr ***(8)***.

2. LabTek chambered coverslip (Nunc, Naperville, IL). Alternatively, chambered coverslips can be made by hand using sterile (tissue-culture treated) polystyrene cell culture dishes with at least 8 cm^2 surface area (35 mm × 10 mm culture dishes), a medium-duty hole punch that can punch a 0.5-inch (1.3 cm) hole, a 15-mL conical tube, silicone vacuum grease, 18 × 18 mm glass coverslips, and 70% ethanol. Use the medium-duty hole punch to punch a 0.5-inch hole in the center of the bottom of a culture dish. Dip a glass coverslip in 70% ethanol and let dry. Dip the top of a 15-mL conical tube into the vacuum grease and then place the inverted tube on top of the hole to create a ring of grease around the hole. Use tweezers to place the ethanolized coverslip over the grease and press on the coverslip to seal it over the hole.
3. Pump with a variable speed drive for accurately dispensing 0.02–1 mL fluid per minute every 6 min, such as the high-precision multi-channel IPC-N 8 pump (ISMATEC, IDEX Corporation, Glattbrugg). Attach 1- mL pipets with 20- to 200-µL pipet tips to the output tubing.
4. 30 m*M* cAMP, pH 6.1. Dissolve cAMP in either of the above Na/K phosphate starvation buffers to achieve a buffered cAMP solution at pH 6.1–6.2. Store in aliquots at –20°C. Stable for 1 yr. When diluting 30 m*M* cAMP for downstream applications, always dilute into one of the starvation buffers to maintain the pH.
5. Micromanipulator (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany). For experiment to experiment reproducibility, commercial micropipets are used (Eppendorf femtotips, New York).
6. Inverted microscope equipped with DIC or phase-contrast optics, a 40× objective lens, and a mercury lamp. If imaging GFP or other fluorescent proteins, the microscope requires filter sets for the relevant wavelengths and either a 63 or 100× oil-immersion objective lens.
7. Charge-coupled device (CCD) camera for acquiring images.
8. Computer with software for controlling the CCD camera and recording images of cell movement, such ImageJ (NIH, Bethesda, MD), IPLab-Spectrum (Scanalytics, Fairfax, VA), or Metamorph (Molecular Devices Corporation, Downingtown, PA).
9. 25 m*M* folic acid, pH 7.0. Dissolve folic acid in either of the above starvation buffers and adjust the pH with $NaHCO_3$ to help it dissolve. Store in aliquots at –20°C. Stable for 1 yr.

2.2. Culture and Preparation of D. discoideum *Cells*

For all chemotaxis applications, *D. discoideum* cells should be taken from axenic exponentially growing or "log-phase" cultures of 1×10^4 to 2×10^6 cells/mL. This can be obtained by shaking cells in nutrient medium, in an autoclaved Erlenmeyer flask, at 150 rpm, 22°C. Determine the cell density by count-

ing using a hematocytometer and calculate how much to dilute the cultures in order to have the necessary number of cells the next day. Alternatively, cells can be expanded in sterile tissue-culture dishes. Because there can be differences in random motility and chemotaxis behavior after growth in shaking culture vs in dishes, it is best to compare the two types of growth when doing chemotaxis assays.

3. Methods

3.1. Analyzing Random Cell Motility

The following protocol is for analyzing unstimulated vegetative cells randomly moving in the absence of chemoattractant.

1. Grow 1×10^6 cells in exponential growth phase in nutrient medium (*see* **Note 1**).
2. Count cell density using a hematocytometer.
3. Seed cells at low density (5×10^4 cells/mL or 2×10^4 cells/cm^2 $\cup$ 1.5×10^5 cells in a 35 mm $\times$ 10 mm, 8 cm^2 chamber) in nutrient medium onto a chambered coverslip (*see* **Note 2**).
4. Allow cells to adhere for 10 min.
5. Wash cells three times in either Na/K phosphate starvation buffer A or B by aspirating the medium/buffer and carefully pipetting buffer onto an inside corner of the dish. Be careful not to disturb the attached cells.
6. Add 3–4 mL of fresh Na/K phosphate starvation buffer (the same buffer used in **step 5**) to cover the cells and let sit for 1 h (*see* **Note 3**).
7. View the cells through a 40× objective (63× or 100× oil-immersion objective if analyzing fluorescent fusion protein localization during random cell motility) on an inverted microscope.
8. Capture images of the cells every 6–12 s for 30–60 min (~300 images) using imaging software and a CCD camera (*see* **Note 4**).

3.2. Analyzing Chemotaxis Toward cAMP

3.2.1. Analyzing Chemotaxis Toward cAMP: Method 1, Developed by the Firtel Laboratory

The following protocol is for analyzing developed cells chemotaxing up a cAMP gradient.

1. Grow 5×10^7 cells in exponential growth phase in nutrient medium (*see* **Note 1**).
2. Count cell density using a hematocytometer.
3. Harvest 5×10^7 cells by centrifuging 5 min at 400*g* (*see* **Note 5**).
4. Wash cells twice by centrifuging 5 min at 400*g* in 40 mL of 12 m*M* Na/K phosphate buffer A (*see* **Note 6**).
5. Resuspend the cells at 5×10^6 cells/mL in 10 mL of 12 m*M* Na/K phosphate buffer A, in a 125-mL Erlenmeyer flask.
6. Make 50 mL of 7.5 µM cAMP by diluting 30 m*M* cAMP into 12 m*M* Na/K phosphate buffer A.

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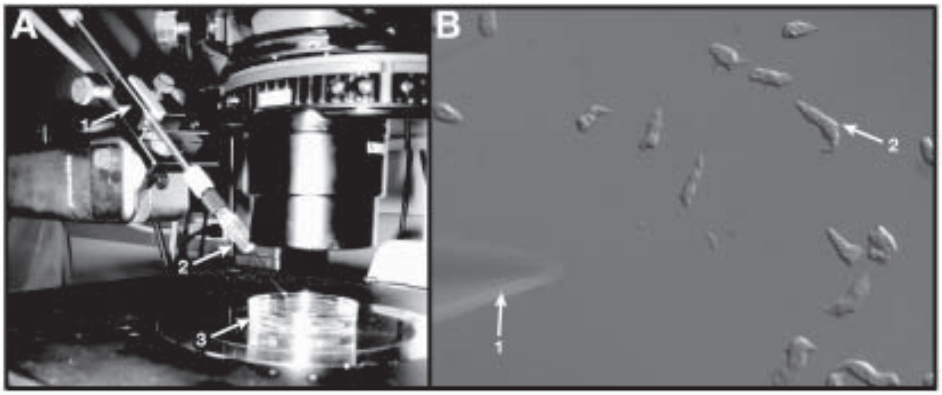

Fig. 2. Positioning a micropipet containing chemoattractant to stimulate *Dictyostelium*. Microscope and micropipet setup. (**A**) A Micromanipulator (1; Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) controls the positioning of the micropipet (2; femtotip, Eppendorf, New York) into the chambered coverslip (3) on the stage of an inverted microscope. (**B**) The needle (1) is positioned at the opposite side of the field as the *Dictyostelium* cells being analyzed (2).

7. Put the input tubing into the 7.5 µ*M* cAMP solution.
8. Calibrate the flow rate of the pump to achieve a 40-µL dispensing volume in 2 s by monitoring the flow output through the 1-mL pipets (*see* **Notes 7** and **8**).
9. Pulse cells with 30 n*M* cAMP at 6-min intervals, for 5 h, by delivering 40-µL pulses of 7.5 µ*M* cAMP into the 10-mL cultures, while shaking at 150 rpm (*see* **Notes 9** and **10**).
10. Fill a chambered coverslip with 12 m*M* Na/K phosphate buffer A and add 75 µL of the pulsed cells (~4×10^6 cells).
11. Allow cells to adhere for 10 min.
12. Place the chamber on the inverted microscope and use the micromanipulator to position a micropipette containing 150 µ*M* cAMP to create a chemoattractant gradient to stimulate the cells (*see* **Notes 11** and **12** and **Fig. 2**). F2
13. Capture a 40× (63× or 100× oil image if analyzing fluorescent fusion protein localization during chemotaxis up a cAMP gradient) image of the cells every 6 s for 30 min (300 images) using imaging software and a CCD camera (*see* **Notes 4** and **13**).
14. Use ImageJ or Metamorph software to convert the stacked TIFF files into a QuickTime (Apple Computer) movie for easier viewing.

3.2.2. Analyzing Chemotaxis Toward cAMP: Method 2, Developed by the Devreotes Laboratory ***(8)***

This protocol differs from that of Method 1 (*see* **Subheading 3.2.1.**) in five ways. The starvation buffer has slightly less phosphate than that of the Firtel

group and includes the addition of $MgSO_4$ and $CaCl_2$. The cells are also starved at a 4X higher concentration and are shaken at a lower rpm during starvation. Starvation begins with 1 h without pulsing, followed by 4 h with pulsing (rather than five straight hours of pulsing). Finally, the cells are pulsed with a higher cAMP concentration and chemotaxis is analyzed with a cAMP gradient created with a 15-fold lower concentration of cAMP, which correlates to a 10-fold decrease in average receptor occupancy.

1. Grow 5×10^7 cells in exponential growth phase in nutrient medium (*see* **Note 1**).
2. Count cell density using a hematocytometer.
3. Harvest 5×10^7 cells by centrifuging at 400*g* for 5 min (*see* **Note 5**).
4. Wash cells twice by centrifuging at 400*g* for 5 min in 40 mL of 10 m*M* Na/K phosphate buffer B (*see* **Note 6**).
5. Resuspend the cells at 2×10^7 cells/mL in 10 mL of 10 m*M* Na/K phosphate buffer B, in a 125-mL Erlenmeyer flask.
6. Shake cells for 1 h at 120 rpm.
7. Make 50 mL of 25 μ*M* cAMP stock by diluting 30 m*M* cAMP into 10 m*M* Na/K phosphate buffer B.
8. Put the input tubing into the 25 μ*M* cAMP solution.
9. Calibrate the flow rate of the pump to achieve a 40-μL dispensing volume in 2 s by monitoring the flow output through the 1-mL pipets (*see* **Note 8**).
10. Pulse cells with 100 n*M* cAMP at 6-min intervals for 4 h by delivering 40-μL pulses of 25 μ*M* cAMP into the 10-mL cultures, while shaking at 120 rpm (*see* **Notes 9** and **10**).
11. Dilute cells to $2 \times \} 10^5$ cells/mL.
12. Spot 20 μL of 2×10^5 cells/mL (4×10^3 cells) onto a chambered coverslip.
13. Allow cells to adhere for 10 min.
14. Cover cells with 10 m*M* Na/K phosphate buffer B to prevent them from drying.
15. Place the chamber on the inverted microscope and use the micromanipulator to position a micropipet containing 10 μ*M* cAMP to stimulate the cells (*see* **Notes 11** and **12** and **Fig. 2**).
16. Capture a 40× (63× or 100× oil image if analyzing fluorescent fusion protein localization during chemotaxis up a cAMP gradient) image of the cells every 6 s for 30 min (300 images) using imaging software and a CCD camera (*see* **Notes 4** and **13**).
17. Use ImageJ or Metamorph software to convert the stacked TIFF files into a QuickTime (Apple Computer) movie for viewing and further analysis.

3.3 Analyzing Chemotaxis Toward Folic Acid: Developed by the Weeks Laboratory (12)

1. Grow 1×10^6 cells in exponential growth phase in nutrient medium (*see* **Note 1**).
2. Count cell density using a hematocytometer.
3. Seed approx 4×10^5 cells/cm^2 onto a chambered coverslip containing either Na/K phosphate starvation buffer A or B.

4. Allow cells to adhere for 10 min.
5. Wash cells once in the Na/K phosphate starvation buffer used in **step 3** by aspirating the medium/buffer and carefully pipetting buffer onto an inside corner of the dish. Be careful not to disturb the attached cells.
6. Add 3–4 mL of fresh Na/K phosphate starvation buffer (the same buffer used in **steps 3** and **5**) containing 20% nutrient medium to cover the cells.
7. Place the chamber on the inverted microscope and use the micromanipulator to position a micropipet containing 25 m*M* folic acid to stimulate the cells (*see* **Note 11** and **Fig. 2**).
8. Capture 40× (63× or 100× oil image if analyzing fluorescent fusion protein localization during chemotaxis up a folic acid gradient) images of the cells every 12–24 s for 1–2 h (300–600 images) using imaging software and a CCD camera (*see* **Note 4**).
9. Use ImageJ or Metamorph software to convert the stacked TIFF files into a QuickTime (Apple Computer) movie for viewing and further analysis.

3.4. Analyzing Cellular Responses to Global Chemoattractant Stimulation

3.4.1. Analyzing Cellular Responses to Global cAMP Stimulation

1. Prepare cells by following **Subheading 3.2.1., steps 1–11** or **Subheading 3.2.2., steps 1–14**
2. Place the chamber on the inverted microscope, focus on a cell of interest, and begin capturing 63× or 100× images every second using imaging software and a CCD camera (*see* **Notes 4** and **14**).
3. Stimulate the cells with 30 µ*M* cAMP by rapidly pipetting 200 µL of 150 µ*M* cAMP per mL of buffer while taking images (*see* **Note 15**).
4. Use ImageJ or Metamorph software to convert the stacked TIFF files into a QuickTime (Apple Computer) movie for viewing and further analysis.

3.4.2. Analyzing Cellular Responses to Global Folate Stimulation

1. Grow 1×10^6 cells in exponential growth phase in nutrient medium (*see* **Note 1**).
2. Count cell density using a hematocytometer.
3. Seed cells at low density (5×10^4 cells/mL or 2×10^4 cells/cm^2 $\cup$ 1.5×10^5 cells in a 35 mm × 10 mm, 8 cm^2 chemotaxis chamber) in nutrient medium onto a chambered coverslip (*see* **Note 2**).
4. Allow cells to adhere for 10 min.
5. Wash cells three times in either Na/K phosphate starvation buffer A or B by aspirating the medium/buffer and carefully pipetting buffer onto an inside corner of the dish. Be careful not to disturb the attached cells.
6. Add 3–4 mL of fresh Na/K phosphate starvation buffer (the same buffer used in **step 5**) to cover the cells and let sit for 1 h (*see* **Note 3**).
7. Place the chamber on the inverted microscope, focus on a cell of interest, and begin capturing 63× or 100× images every second using imaging software and a CCD camera (*see* **Notes 4** and **14**).

8. Stimulate the cells with 50 μ*M* folic acid by rapidly pipetting 100 μL of 500 μ*M* folic acid per mL of buffer while taking images (*see* **Note 15**).
9. Use ImageJ or Metamorph software to convert the stacked TIFF files into a QuickTime (Apple Computer) movie for viewing and further analysis.

4. Notes

1. When growing cells, it is important to take into account that wild-type cells double every 8 h, but mutant strains can take longer. For example, shaking 1×10^7 cells (about one confluent 10-cm plate) in 20 mL of nutrient medium overnight (16 h or two doubling periods) usually yields about 4×10^7 cells at 2×10^6 cells/mL the next morning. Furthermore, some mutant strains, such as strains with inactive myosin II, exhibit conditional cytokinesis defects when grown in shaking cultures ***(17,18)***. These cells become very large and multinucleate as a result of a lack of cell division in the absence of a solid substratum. For chemotaxis analyses, such strains should always be expanded on sterile tissue culture dishes rather than in shaking cultures.
2. The low cell density facilitates tracking individual cell movements within the field of view over the course of the assay.
3. Random motility is assayed in Na/K phosphate buffer, rather than nutrient medium, because nutrient medium contains folic acid, a chemoattractant. However, after >2 h in Na/K phosphate starvation buffer, *Dictyostelium* secretes pulses of cAMP, another chemoattractant. Thus, less than 2 h should elapse from the point of addition of starvation buffer to the end of analysis. To ensure that no autonomous chemoattractant signaling is occurring, seed the cells on and monitor their motility in a perfusion chamber, such as a Dvorak-Stotler chamber (Nicholson Precision Instruments, Gaithersburg, MD) or a Sykes-Moore chamber (Bellco Glass, Vineland, NJ) ***(3)***. Connect the inlet tube of the chamber to a reservoir containing Na/K starvation buffer and the outlet tube to a peristaltic pump in order to replace the chamber fluid with fresh buffer every 8 s ***(19)***.
4. If using fluorescent probes to monitor protein localization or activity during chemotaxis, exposure times and/or exposure frequency may need to be reduced to avoid photobleaching the cells. Fluorescent proteins are sensitive to ultraviolet (UV) light, and the microscope system and camera sensitivity are important. For example, using a Nikon model TE300 microscope and Coolsnap-HQ camera, images with 50- to 150-ms exposure times can be taken every second using a shutter system. For faster (<1 s) images, such as those taken during global stimulation, the shutter will need to remain permanently open. Although this exposes the cells to more UV light, the total recording time is shorter for these applications (5 min for global stimulation vs 30 min for chemotaxis analysis).
5. If sequentially analyzing multiple strains, it is best to re-count, wash, and begin pulsing the different strains at 1-h intervals to ensure that each strain is starved for the same amount of time before analysis. Analyzing each strain for chemotaxis toward cAMP entails recording the cells' movement for 30 min, and two recordings may be desired for internal controls and downstream analysis. Some-

times, inadvertently starving the cells for a longer time before analysis can result in more polarized cells that move faster and with better directionality towards cAMP.

6. It is important to ensure that the *Dictyostelium* cells are washed free of nutrients found in the nutrient medium before setting up the starvation program. Be sure to sufficiently dilute the cells in the Na/K starvation buffer when washing.
7. The key is to be able to deliver 30 n*M* pulses of cAMP to the cells without drastically altering the volume (and thus the concentration) of the cells. Pulsing 10 mL of cells at 5×10^6 cells/mL with 40-µL pulses adds a total volume of 2 mL after 5 h and dilutes the cells to approx 4×10^6 cell/mL. The conditions can be adjusted to deliver 20-µL pulses of 15 µ*M* cAMP to add only 1 mL total volume over the course of the experiment. However, pulsing less than a 20-µL volume can cause problems with the pulse that can lead to the cAMP drop not having enough weight to counter the surface tension and falling into the shaking flask. Pulsing larger than a 40-µL volume will dilute the cells too much. In the absence of a pump, cAMP can be manually added to the cells every 6 min or, if it is known that the strains are capable of secreting and responding to cAMP, they can be incubated in the chambered coverslip for 6–8 h and allowed to develop on their own.
8. It is critical to check the tubing and pipets for bubbles and leaks. If pulsing more than one strain at a time, each input/output tubing combination needs to be calibrated to ensure that there is ≤10% difference in the amounts of cAMP delivered to the individual strains.
9. Wild-type cells should be elongated (polarized) after proper cAMP pulsing.
10. Some protocols call for a 30-min treatment with caffeine after pulsing and before the actual chemotaxis analysis, in order to inhibit adenylyl cyclase and reduce any basal chemotaxis signaling. However, this method is problematic and unadvisable because caffeine likely specifically and nonspecifically inhibits many other cellular activities. For example, it has recently been discovered that caffeine inhibits TOR Complex 2, which among many other substrates, phosphorylates and activates Akt and is required for chemoattractant-mediated activation of adenylyl cyclase (Lee and Firtel, unpublished data) ***(20,21)***. Akt is a key signaling protein required for actin polymerization and pseudopod protrusion and its inhibition would severely affect the chemotaxis phenotype ***(6)***.
11. In order to provide the maximum distance for the cells to travel before reaching the micropipet tip, position the micropipet at the edge of the field of view opposite to that of the cells to be analyzed.
12. The cAMP gradient generated with 1–10 µ*M* cAMP has been estimated to create a 2–10% change in receptor occupancy across the cell ***(8)***, the minimum gradient detected by eukaryotic cells. In the methods described here, cAMP diffuses from the tip of the micropipet. Other methods use positive pressure. In these cases, a lower concentration of cAMP is used in the micropipette.
13. Mutant strains that do not polarize after pulsing and/or do not chemotax toward the micropipet must be checked for expression of the cAMP receptors (cAR1 and cAR3) and other signaling proteins normally induced by cAMP pulsing and

necessary for adenylyl cyclase activation, such as PKA, Erk2, and Myb ***(9,10)***. Either Northern blot analysis and/or immunoblotting for expression of such genes/proteins will determine whether the mutation blocks a chemoattractant-driven event or the developmental transition into chemotaxis competence.

14. Some signaling events occur faster than others and the frequency of recorded images should be adjusted accordingly. For example, GFP-RBD translocation is faster than that of GFP-PH, and images must be taken every 0.2 s to differentiate the two responses ***(15)***.
15. A flow chamber with inlet and outlet tubes can also be used to globally stimulate cells with a uniform concentration. Both input and output tubes are filled with Na/K buffer to the same level. Fresh buffer containing the chemoattractant of interest is then added to the inlet tube, causing the chamber to fill with the chemoattractant by laminar flow without creating a gradient, in the course of approx 1 s ***(14,22)***.

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