Video Article

Assessment of *Dictyostelium discoideum* Response to Acute Mechanical Stimulation

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URL: https://www.jove.com/video/56411
DOI: doi:10.3791/56411

Keywords: Cellular Biology, Issue 129, Signal transduction, mechanotransduction, shear stress, chemotaxis, rheotaxis, shear flow-induced migration, directed migration, biosensor localization, acute stimulation

Date Published: 11/9/2017

Citation: Artemenko, Y., Devreotes, P.N. Assessment of *Dictyostelium discoideum* Response to Acute Mechanical Stimulation. *J. Vis. Exp.* (129), e56411, doi:10.3791/56411 (2017).

Abstract

Chemotaxis, or migration up a gradient of a chemoattractant, is the best understood mode of directed migration. Studies using social amoeba *Dictyostelium discoideum* revealed that a complex signal transduction network of parallel pathways amplifies the response to chemoattractants, and leads to biased actin polymerization and protrusion of a pseudopod in the direction of a gradient. In contrast, molecular mechanisms driving other types of directed migration, for example, due to exposure to shear flow or electric fields, are not known. Many regulators of chemotaxis exhibit localization at the leading or lagging edge of a migrating cell, as well as show transient changes in localization or activation following global stimulation with a chemoattractant. To understand the molecular mechanisms of other types of directed migration we developed a method that allows examination of cellular response to acute mechanical stimulation based on brief (2 - 5 s) exposure to shear flow. This stimulation can be delivered in a channel while imaging cells expressing fluorescently-labeled biosensors to examine individual cell behavior. Additionally, cell population can be stimulated in a plate, lysed, and immunoblotted using antibodies that recognize active versions of proteins of interest. By combining both assays, one can examine a wide array of molecules activated by changes in subcellular localization and/or phosphorylation. Using this method we determined that acute mechanical stimulation triggers activation of the chemotactic signal transduction and actin cytoskeleton networks. The ability to examine cellular responses to acute mechanical stimulation is important for understanding the initiating events necessary for shear flow-induced motility. This approach also provides a tool for studying the chemotactic signal transduction network without the confounding influence of the chemoattractant receptor.

Video Link

The video component of this article can be found at https://www.jove.com/video/56411/

Introduction

Migration of eukaryotic cells is biased by diverse chemical and physical cues in the environment, including gradients of soluble or substrate-bound chemoattractants, variable stiffness of the substrates, electric fields, or shear flow. Although there have been many advances in our understanding of the molecular mechanisms driving chemotaxis, less is known about other types of directed migration and how these diverse signals are integrated at the cellular level to produce a unified migratory response.

Directed migration toward an increasing concentration of a chemoattractant involves three behavioral components: motility, directional sensing, and polarity[1]. Motility refers to random movement of cells achieved by pseudopod protrusion. Directional sensing is the ability of a cell to detect the source of a chemoattractant, which can occur even in immobilized cells. Polarity refers to the more stable asymmetrical distribution of intracellular components between the leading and lagging edge of a cell, which leads to increased persistence in movement.

Cellular response to a chemoattractant depends on the activity of four conceptually defined regulatory networks: receptor/ G protein, signal transduction, actin cytoskeleton, and polarity[1]. Chemoattractant binding to the G protein-coupled receptor transmits the signal via heterotrimeric G proteins α and βγ to the downstream signal transduction network, which amplifies the directional signal. Multiple pathways within the signal transduction network act in parallel and feed into the actin cytoskeleton network to bias actin polymerization, and consequent pseudopod protrusion, in the direction of the gradient. Among important regulators of chemotaxis are Ras GTPase, TorC2, phosphoinositide 3-kinase (PI3K), phosphatase and tensin homolog (PTEN), and guanylyl cyclase. Feedback mechanisms within the signal transduction network and between the signal transduction and actin cytoskeleton networks further amplify the response. Finally, the poorly defined polarity network receives input from the actin cytoskeleton, and further biases the signal transduction network to promote persistent migration in the direction of the gradient.

Much of our mechanistic understanding of chemotaxis was made possible because of the development of fluorescently-tagged biosensors for various components of the regulatory networks. Many chemotaxis regulators have an asymmetrical distribution either of the regulatory molecule itself or its activity. For example, biosensors that recognize activated versions of small GTPases Ras and Rap1 – Ras-binding domains of Raf1 (referred to as RBD here) and RalGDS, respectively – localize to the leading edge of a chemotaxing cell[2,3]. Similarly, PI3K and its product

phosphatidylinositol (3,4,5)-trisphosphate (PIP3), recognized by a pleckstrin homology (PH) domain, also show localization at the front of a cell[4,5]. In contrast, a 3-phosphatase PTEN, which converts PIP3 back to phosphatidylinositol (4,5)-bisphosphate, localizes to the lagging edge of the cell[6]. Importantly, these biosensors change their localization in response to global stimulation with a chemoattractant. Leading edge markers, which are cytosolic or on the tips of protrusions in a resting cell, relocalize to the cortex, whereas lagging edge markers, which have cortical localization and are absent from the tips of protrusions in a resting cell, become cytosolic after stimulation. Analysis of biosensor distribution in response to global stimulation with a chemoattractant minimizes the contribution of motility and polarity, which often confound the observations. Global or uniform stimulation of a cell suspension with a chemoattractant is also used as a tool to assess population-wide changes in protein activation, often detected by protein phosphorylation[7,8,9]. This biochemical assay is primarily used to obtain temporal information, whereas microscopy is used to gather both temporal and spatial information about the behavior of various components of the regulatory networks.

The signal transduction network incorporates features of an excitable system[10,11]. Responses to supra-threshold chemotactic stimuli are "all-or-none" and display refractory periods. Responses are also triggered stochastically and can show oscillatory behavior. Signal transduction events are localized to regions of the cortex that propagate as waves[12,13,14,15]. Front, or back, markers are recruited to, or dissociate from, the active zones of the propagating waves. Due to the refractory region trailing the active zone, the oppositely directed waves annihilate as they meet. The propagating signal transduction waves underlie the cellular protrusions that mediate cell migration[10].

Much of the aforementioned information on chemotaxis came from the studies on the social amoeba *Dictyostelium discoideum*, although similar regulatory mechanisms are also applicable to neutrophils and other mammalian cell types[16]. *Dictyostelium* is a well-established model organism that has a robust chemotactic response during starvation, when thousands of single cells migrate toward an aggregation center, eventually forming a multicellular fruiting body containing spores. Chemotaxis is also essential during the single-cell growth stage of this organism for locating bacterial food sources. Importantly, migration of single *Dictyostelium* cells is remarkably similar to the migration of mammalian neutrophils or metastatic cancer cells, all of which undergo very rapid amoeboid-type migration. In fact, both the overall topology of the regulatory networks, as well as many of the individual signal transduction pathways involved in chemotaxis are conserved between *Dictyostelium* and mammalian leukocytes[17]. Furthermore, other cells, such as fibroblasts, use receptor tyrosine kinases (RTK) instead of GPCRs; however, RTKs may feed into similar networks.

In contrast to chemotaxis, thorough understanding of the signaling mechanisms that drive various other modes of directed migration is lacking. Similarly to cells migrating in a chemoattractant gradient several studies have reported activation and/or localization of typical leading edge markers, including actin polymerization, PIP3 and/or extracellular signal-regulated kinase (ERK) 1/2, at the front of cells undergoing directed migration in response to shear flow or changes in electric fields[18,19,20,21]. However, in these studies continuous exposure to the stimulus also resulted in cell migration, leaving open the question whether, for example, the leading edge markers localize specifically in response to a stimulus, or if they simply localize at the leading edge because of increased number of pseudopods at the front of a migrating cell.

We developed assays that allow us to observe the response of cells to acute mechanical perturbation delivered as shear flow both at the population level and as individual cells[22]. Similar to global stimulation with a chemoattractant, acute stimulation with shear flow allows the study of a cellular response to a mechanical stimulus without the confounding contribution from motility or polarity. Combining these biochemical and microscopic assays with genetic or pharmacological perturbations allows us to learn about how mechanical stimuli are perceived and transmitted. Moreover, this approach also provides a novel method for tapping into the system downstream of the chemoattractant receptor in the absence of a chemoattractant, thereby isolating the signal transduction and actin cytoskeleton networks from the receptor/G protein network.

Using the techniques described below we recently demonstrated that acute shear stress leads to activation of multiple components of the chemotactic signal transduction and actin cytoskeleton networks[22]. By applying the acute mechanical stimulus at varying intervals, we demonstrated that, similarly to chemoattractants, response to mechanical stimuli also exhibits features of an excitable system, including the all-or-none behavior of the response under saturating conditions and the presence of a refractory period. Finally, by combining mechanical and chemical stimulation we showed that the two stimuli share signal transduction and actin cytoskeleton networks, which likely allow for integration of multiple stimuli to bias cell migration.

Protocol

1. Preparation of Solutions

1. Prepare HL-5 media using the following: 10 g/L dextrose, 10 g/L proteose peptone, 5 g/L yeast extract, 0.965 g/L $Na_2HPO_4 \cdot 7H_2O$, 0.485 g/L KH_2PO_4, and, unless otherwise indicated, 0.03 g/L streptomycin in deionized water. Autoclave the medium and store at room temperature.
   NOTE: Due to differences between proteose peptone and yeast extract acquired from different suppliers, as well as between individual batches, pH of the media may need to be adjusted to the typical range of 6.4 to 6.7.
2. Prepare SM plates using the following: 10 g/L dextrose, 10 g/L peptone, 1 g/L yeast extract, 2.31 g/L KH_2PO_4, 1 g/L K_2HPO_4, and 20 g/L agar in deionized water. Autoclave, cool, and pour 30 mL of the agar solution per 10-cm Petri dish. Once the agar solidifies, store the plates at 4 °C for up to 1 month.
3. Prepare 10x Phosphate Buffer (PB) using 13.4 g/L $Na_2HPO_4 \cdot 7H_2O$ and 6.8 g/L KH_2PO_4 in deionized water. Store the 10x stock at 4°C. Dilute to 1x with deionized water for use.
4. Prepare DB Buffer by supplementing 1X PB with 2 mM $MgSO_4$ and 0.2 mM $CaCl_2$.
5. Prepare 100 mM caffeine in deionized water. Store at −20°C.
6. Prepare 100 mM cAMP stock solution by dissolving cAMP sodium salt monohydrate in deionized water. Prepare a 1 mM working stock in deionized water. Store both stock solutions at −20 °C. Prepare final cAMP solution at the desired concentration in DB on the day of the experiment.
   NOTE: Stock solutions can be freeze-thawed a few times.
7. Prepare 25 mM folic acid in 1x PB. Add a few drops of 1 N NaOH until the powder completely dissolves. Store at −20 °C.
8. **Prepare 3x sample buffer using the following: 187.5 mM Tris-HCl pH 6.8, 6% (w/v) sodium dodecyl sulfate (SDS), 30% glycerol, 126 mM dithiothreitol, and 0.03% (w/v) bromophenol blue. Aliquot and store at −20 °C.**

1. Prior to use, thaw in a 37 °C water bath and proceed to prepare sample buffer with protease and phosphatase inhibitors by diluting 3x sample buffer to 1x, and adding 50 mM NaF, 2 mM Na_3VO_4, 25 mM sodium pyrophosphate, and 1x complete EDTA-free protease inhibitor cocktail in deionized water. Add the inhibitors immediately before starting the procedure described in steps 3.1.2-3.1.3.

9. Prepare 5 mM Latrunculin A solution in DMSO. Aliquot and store at −20°C.

2. Preparation of *D. discoideum* cells

NOTE: Maintain *D. discoideum* cells in HL-5 media either in tissue culture plates or in a suspension culture as previously described[23]. When necessary, transform cells with fluorescently-labeled biosensors according to standard electroporation protocols[24]. Various *D. discoideum* strains, as well as plasmids encoding biosensors or other genes of interest may be obtained from the Dicty Stock Center (dictybase.org).

1. **Growth and Collection of Vegetative *D. discoideum* Cells**
   1. For analysis of vegetative cells, grow cells in the presence of bacteria to reduce the number of macropinosomes, which are typically observed in axenically-grown cells[25].
      1. Prepare a culture of *Klebsiella aerogenes* by inoculating a small number of cells from a glycerol stock or from a previous culture into the desired volume of HL-5 medium without antibiotics. Incubate bacterial culture on an orbital shaker at 200 rpm (0.42 x g) at room temperature (20-22 °C) for 16-18 h.
         NOTE: The *K. aerogenes* strain used here is non-pathogenic (see the **Table of Materials**).
   2. Collect *D. discoideum* in the exponential growth phase either from tissue culture plates or from a suspension culture, and count the cells using a hemocytometer according to manufacturer's instructions. Spread 1 x 10^5 *D. discoideum* cells with 260 µL *K. aerogenes* suspension on an SM plate. Turn the plate upside down the next day. Grow cells at room temperature until the *D. discoideum* cells start clearing the bacterial lawn but before they begin aggregating (~36-48 h).
   3. Collect *D. discoideum* cells in 5 mL DB buffer by scraping them with a glass spreader. Transfer cells to a 50 mL polypropylene tube. Rinse the plate with another 5 mL DB buffer and pool with the original suspension. Fill the tube to 50 mL with DB buffer.
   4. Centrifuge the *D. discoideum* suspension at 360 x g for 3-4 min. Aspirate the supernatant and resuspend the pellet in 50 mL DB Buffer. Repeat the washing steps until the supernatant is clear (~3 to 4 washes). Resuspend the final cell pellet in DB buffer to a final density of ~5 x 10^6 cells/mL.

2. **Preparation of Aggregation-Competent *D. discoideum* Cells**
   1. Develop *D. discoideum* cells by 1 h starvation followed by 4 h of starvation and pulsing with 50 nM cAMP every 6 min according to standard protocols[23].
   2. Following development, measure the final volume of cell suspension.
   3. Based on the initial number of cells used for development, calculate cell density of the final suspension.
      NOTE: If cAMP is delivered in 100 µL volumes every 6 min, cell volume increases by 1 mL for every hour of cAMP pulsing. Thus, for the standard conditions using 8 x 10^7 cells in 4 mL of DB, after 4 h of development the final volume is 7 mL and the final cell density is ~1.1 x 10^7 cells/mL.

3. Biochemical Analysis of Cell Response to Mechanical or Chemical Stimulation

1. **Acute Mechanical Stimulation of Cells Followed by Cell Lysis**
   1. Plate 2 x 10^6 aggregation-competent cells (from step 2.2) after 4 h of development in 35-mm dishes with 2 mL DB. Allow the cells to attach for 10 min at room temperature. Wash cells twice with 1 mL DB. Incubate cells in DB with 2.5 mM caffeine for 30 min without any disturbance of the plates.
      NOTE: If cells need to be treated with a pharmacological inhibitor, add desired concentration of inhibitor or the same volume of appropriate vehicle during basalation with caffeine.
   2. To apply mechanical stimulation, place the plate (one at a time) on an orbital shaker and immediately turn it on at 150 rpm (0.24 x g) for 5 s.
      NOTE: The plate can also be manually stimulated by movement in a cross-wise manner for approximately 5 s.
   3. Aspirate the buffer at the indicated times after the start of the stimulation. Immediately lyse the cells by adding 100 µL sample buffer with protease and phosphatase inhibitors.
   4. Place the plate on ice, and collect the lysate into a 1.5-mL tube. For 'time 0', lyse the cells without shaking. Transfer the tubes to a 95 °C heat block for 10 min immediately after lysis. Proceed with immunoblotting or store lysates at -20 °C.

2. **Stimulation of Cells with a Chemoattractant**
   1. Basalate aggregation-competent cells after 4 h of development by rapidly shaking them in the presence of 5 mM caffeine at 200 rpm (0.42 x g) on an orbital shaker for 30 min.
      1. Centrifuge the cell suspension at 360 x g for 3-4 min. Aspirate the supernatant and resuspend the pellet in 10 mL ice-cold DB Buffer. Repeat the washing step.
   2. Resuspend the final cell pellet in ice-cold DB buffer to a final density of 4 x 10^7 cells/mL based on the initial cell number used to develop the cells (see step 2.2). Keep the cell suspension on ice prior to stimulation.
      1. Pipette 50 µL of 10 µM cAMP or vehicle into a polystyrene cup placed on an orbital shaker.
   3. To stimulate the cells, add 450 µL of the ice-cold cell suspension into the same cup and immediately turn the shaker on at 200 rpm (0.42 x g). At various times after the start of the stimulation (*e.g.* 10, 30, 45, 60, 120 s), briefly stop the shaker, take out 50 µL aliquots of cell suspension, and lyse the cells by adding them to 1.5 mL tubes containing 25 µL 3X sample buffer.
      1. For 'time 0', use the cells from the unstimulated ice-cold cell suspension.

NOTE: The final temperature of the cell suspension during stimulation is ~9 °C[26]. Under these conditions the peak response occurs slightly later than the peak observed for stimulation performed at room temperature (for example, chemoattractant stimulation of adherent cells on the microscope, or mechanical stimulation of adherent cells).

4. Transfer the tubes to a 95 °C heat block for 10 min immediately after lysis. Proceed with immunoblotting or store lysates at -20 °C.

3. **Analysis of Cell Response by Immunoblotting**
    1. Run lysates (from steps 3.1.3 or 3.2.4) on a 4-15% Tris-HCl polyacrylamide gel, transfer to a polyvinylidene fluoride membrane, block, and immunoblot with phospho-PKCζ Thr410 (to detect phospho-PKBR1 and phospho-PKBA). Detect the signal by incubation with horseradish peroxidase-conjugated anti-rabbit secondary antibody, followed by chemiluminescence using enhanced chemiluminescence substrate.
    2. For detection of multiple proteins, strip the blot with stripping buffer, and re-probe with a primary antibody against phospho-p42/44 MAPK Thr302/304 (to detect phospho-ERK2). Confirm equal protein loading by staining the polyvinylidene fluoride membrane with Coomassie Brilliant Blue.

4. Acute Mechanical Stimulation and Live Imaging of Single Cells on the Microscope

1. **Assessment of the response to acute mechanical stimulation using a flow device**
    1. Collect and dilute vegetative or aggregation-competent cells to ~1 x 10^6 cells/mL in DB as described in steps 2.1 and 2.2, respectively. Load ~600 µL into the slide with a channel (see the **Table of Material** for details). Allow cells to attach for 10 min. Make sure that all of the inlets in the slide are completely filled. Top up with extra buffer if necessary.
    NOTE: The particular slide used for analysis has three inputs, which feed into narrow, 1-mm channels, but then merge to one wide, 3-mm channel. The height of the channel is 0.4 mm. Although the cells are plated throughout the channel, only the wide portion is imaged.
    2. Pass one of the lines that is attached to a 50-mL reservoir through the front right valve of the fluidic unit (see the **Table of Materials** for details). Using the software for the pump, make sure the valve is closed (*i.e.* to the left). Fill the reservoir with DB.
        1. Set the pressure at 50 mbar and turn it ON. Fill and rinse the line with DB by clicking on the valve to open it. Turn the pressure back to 0, and close the valve after ~30 s.
    3. Connect the line to one of the three inlets on one side of the slide without trapping any air bubbles. Plug the other two inlets. Connect the line from the drain to the single inlet on the second side of the slide.
    NOTE: The tubing used for the input and drain lines in this setup has an internal diameter of 1.6 mm.
    4. Place the slide on the microscope under a 20X air objective. To rinse the channel, switch the valve to open the line and click "Pressure ON", starting at higher pressure (~50 mbar or ~40 dyn/cm^2) to push the liquid through to the drain.
        1. Once the liquid comes out of the drain, reduce external pressure to zero (*i.e.* gravity flow only, ~15 dyn/cm^2), and continue rinsing for ~30 s. Switch the valve to the opposite position to stop the flow.
    5. Acquire images with RFP or GFP illumination on an inverted fluorescence microscope under a 40X oil objective at 3 s intervals. With the valve in the closed position, turn the pressure ON at the desired pressure, typically between 15 and 40 dyn/cm^2 (0 - 50 mbar).
    6. After acquiring 5 frames, deliver the stimulus by switching the valve to the "open" position. Turn the flow off after 2-5 s by switching the valve to the opposite direction.
    NOTE: For certain weaker biosensors (*e.g.* PTEN, CynA, and RalGDS) it may be necessary to image cells using a confocal microscope equipped with a 40X oil objective.

2. **Testing effects of pharmacological inhibitors on response to acute mechanical stimulation using a flow device**
    1. Plate cells in the slide with a channel as described in step 4.1.1. Set up two lines: pass one line through the front right valve as in step 4.1.2, and the second through the back left valve. Clamp the left line and put the valve in the "closed" (left) position. Fill one line with DB containing the appropriate vehicle, and the second with the solution containing a pharmacological inhibitor of interest (*e.g.* 5 µM Latrunculin A).
    NOTE: It is necessary to physically clamp the left line because the "closed" left position opens the valve for that line.
    2. Fill and rinse the right line by turning the pressure ON at 50 mbar and switching the valve to the "open" (right) position. Switch the valve to the left after ~30 s. Fill and rinse the left line by removing the clamp for ~30 s. Connect both lines to two of the inlets on one side of the slide with a channel, plug the remaining inlet, and connect the drain to the single inlet on the second side.
    3. Wash the slide with the buffer in the right line and perform stimulation in the same buffer as described in steps 4.1.3 and 4.1.4 above.
    NOTE: Although the right line was chosen as the first one in this setup, the order could be reversed.
    4. Following stimulation, switch the valve to open the right line at zero pressure (*i.e.* gravity flow only, ~15 dyn/cm^2). Remove the clamp from the left line and switch the valve to the left to open that line. Clamp the first line.
    5. After running 3-5 mL of buffer with inhibitor through, switch the valve to the right to stop the flow, and incubate the cells with the inhibitor for the required length of time (*e.g.* 15 min). Turn the flow on by switching the valve every ~10 min for ~15 s to prevent oxygen deprivation. Repeat stimulation with the second line.

3. **Alternative method to assess response to acute mechanical stimulation using a micropipette**
    1. Set up the micropipette filled with DB according to standard protocol[23]. Keep the compensation pressure at 1,500 psi.
    2. Collect and dilute vegetative cells as described in step 2.1. Place 25 µL drops of ~7.5 x 10^5 cells/mL in a 1-well chamber, allow to adhere for at least 10 min, and cover with 3 mL DB.
    3. Place the chamber on an inverted fluorescence microscope equipped with a 40X oil objective. Locate the cells. Gently lower the micropipette into the middle of the field of view until it first touches the bottom of the chamber.
    NOTE: Since the compensation pressure was set at 1,500 Psi, the cells will be continuously exposed to a very slow flow of DB from the micropipette.

4. Begin acquiring images with RFP or GFP illumination at 3 s intervals. Apply the 'Clean' function to release a bolus of liquid from the micropipette. Continue imaging in the presence of flow due to compensation pressure alone.

4. **An alternative method to assess response to acute mechanical stimulation using bulk buffer addition**
    1. Collect and dilute vegetative cells as described in step 2.1. Place 20 µL drops of cells at ~1 x 10^6 cells/mL in DB in the middle of a well from an 8-well chamber. Allow cells to adhere for at least 10 min.
    2. Begin acquiring images with RFP- or GFP-specific illumination on an inverted fluorescence microscope equipped with a 40X objective at 3 s intervals. Focus on an area close to the edge of the drop.
    3. Rapidly add 430 µL of DB to one side of the well. Continue imaging.
    4. For analysis of the interaction between mechanical and chemical stimuli, 12 or 45 s following mechanical stimulation, gently add 50 µL of folic acid (final concentration 20 nM) or vehicle (DB) without inducing a mechanical response. Continue imaging.

5. **Quantification of Response**
    1. Open the 32-bit TIFF image in the Image Analysis Software (see **Table of Materials** for details)[27].
    2. Under the 'Analyze' tab, go to "Set Measurements". Check the box for 'Mean Gray Value'. Make sure the other boxes are not checked. Click "OK".
    3. Under the 'Process' tab, click "Subtract Background". Keep the rolling ball radius at 50 pixels, and do not check any of the options listed in the menu. Zoom in on one cell using the 'Magnifying Glass' tool.
    4. Using the 'Rectangle' tool, draw a box (~2.5 x 2.5 µm^2) in the cytosol, making sure not to draw it over the nucleus or the plasma membrane. Press "Ctrl" + "M" keys or go to the 'Analyze' tab and click on "Measure" to determine the mean gray value of the box. Advance to subsequent frames and measure again, making sure the box stays in the cytosol.
    NOTE: Since *D. discoideum* cells move very rapidly the box can be moved from one frame to the next to keep it in the cytosol.
    5. After all of the frames have been analyzed, copy the values into a spreadsheet. To account for cell-to-cell variation in the expression levels of various biosensors, normalize the values for the mean gray value observed at time 0. Calculate the reciprocal of the values to reflect the accumulation of the signal on the cortex.

5. Continuous Mechanical Stimulation and Live Imaging of Single Cells on the Microscope

1. Set up the cells exactly as described above for acute mechanical stimulation analysis in steps 4.1.1 - 4.1.3. Open the plug covering the reservoir with the buffer so the volume can be topped if response is analyzed for longer than a few minutes at a time.
NOTE: Because the reservoir remains open for the duration of the experiment, this assay is conducted in the absence of external pressure and relies on gravity flow alone. To increase the rate of flow by gravity, the drain can be placed on a table below the microscope.
2. Begin imaging cells with RFP- or GFP-specific illumination on an inverted fluorescence microscope equipped with a 40X objective at 3 s intervals for analysis of biosensor responses. Alternatively, image with phase contrast using a 20X objective at 10 s intervals for analysis of overall cell migration.
3. Turn on the flow after several frames at the zero-pressure setting (*i.e.* flow is due to gravity alone or ~15 dyn/cm^2) by switching the valve to the open position. When the level of fluid falls to 5-10 mL in the reservoir, top up with more buffer. Make sure to add the fluid carefully so air bubbles do not get trapped in the lines.
NOTE: It is important to add fluid of the same temperature to avoid a shock response in the cells.
4. Quantify cell speed and persistence using migration analysis software (see the **Table of Materials** for details) according to manufacturer's instructions.

Representative Results

Temporal response of chemotaxis regulators to acute mechanical stimulation

To assess the response of *D. discoideum* cells to mechanical stimulation, adherent aggregation-competent cells were exposed to a brief pulse of shear flow. Aggregation-competent *D. discoideum* cells secrete cAMP, which can be sensed by neighboring cells. To overcome the contribution of cell-cell signaling, cells were treated with caffeine, which inhibits adenylyl cyclase in *D. discoideum* and thus prevents cAMP secretion[28]. Indeed, cells pre-treated with caffeine had very low basal activity of PKBR1 and ERK2, and showed a robust increase in the phosphorylation of the key residues of these kinases following 5 s stimulation with shear flow (**Figure 1A**). The response was transient and peaked around 10-15 s for PKBR1, and around 30 s for ERK2, similarly to previously published findings for activation of these proteins with a chemoattractant[7].

Although it is difficult to evaluate the efficiency of the response considering the low basal level, preliminary studies that led to the development of this assay, compared stimulation with shear flow alone (or with a vehicle) to shear flow in the presence of a chemoattractant cAMP (**Figure 1B**). This analysis did not show an increase in the response with the cAMP, suggesting that the response of the signal transduction network to shear flow is saturated. Although in this assay cAMP did not increase phosphorylation of PKBR1, cells' response to chemoattractant can be observed using a standard stimulation protocol where aggregation-competent cells are kept in suspension, stimulated with cAMP, and lysed at various time points following stimulation. As shown in **Figure 1C**, under these conditions there is a transient increase in PKBR1 phosphorylation in response to chemoattractant, but not vehicle. The peak response is observed at 30 s in this assay because the temperature of the cell suspension during the assay is ~9 °C, compared to ~22 °C for mechanical stimulation assay described above[26].

Spatiotemporal response of leading and lagging edge markers to acute mechanical stimulation

Since the population assay above only provides temporal information about the behavior of chemotaxis regulators, cells were also exposed to a brief mechanical stimulus while being observed with a microscope. This assay can be performed either with aggregation-competent or vegetative *D. discoideum* cells expressing various fluorescently-labeled biosensors whose localization was defined in cells stimulated with a chemoattractant. Two leading edge markers, PH domain of Crac and $LimE_{\Delta coil}$, which are recruited by PIP3 or newly-polymerized actin, both showed mostly cytosolic localization in resting cells as previously reported (**Figure 2A**, **Supplemental Movie 1**)[4,29]. In cells that were basally active, these biosensors could also be seen on the tips of the pseudopods. Following stimulation with shear flow for 2 s, the biosensors rapidly relocalized to the cortex with a peak at ~6 to 10 s, and returned to the cytosol by ~30 s. In contrast, a lagging edge marker PTEN localized at the cortex before stimulation (**Figure 2A**). After a brief pulse with shear flow, cytosolic PTEN intensity increased, albeit with slower dynamics than for the leading edge biosensors. A change in biosensor localization from the cytosol to the cortex, and vice versa, is clearly seen as a change in the cytosolic intensity allowing for simple quantification of the response.

The observed behavior of the biosensors in response to acute mechanical stimulation is consistent with leading and lagging edge localization dynamics in response to stimulation with uniform chemoattractant. This behavior was not unique to the three biosensors shown. As previously published, similar changes in localization were also observed for leading edge markers RBD and RalGDS, which recognize activated Ras and Rap1, respectively, as well as for another lagging edge marker CynA[22,30].

A similar assay can be used to assess the effects of various inhibitors by a simple modification of the experimental setup from a single input line to a double one. Using this method, the cells can be first exposed to control conditions, and then switched to the buffer that contains the desired test agent. For example, addition of actin-depolymerizing drug LatA blocked the response of RBD, as well as $LimE_{\Delta coil}$, to acute mechanical stimulation (**Figure 2B**).

Global response precedes cell migration under prolonged stimulation with shear flow

The approach described here could also be adapted to study effects of shear flow on *D. discoideum* migration. In fact, if the flow was not shut off after 2 s but remained on for the duration of the experiment, vegetative cells showed directed migration against the flow (**Figure 3, Supplemental Movie 2**). It should be noted that the shear flow necessary to elicit a migratory response was lower than the pressure that was typically used to achieve a global response with acute stimulation in vegetative cells (for example, as seen in **Figure 2B**). However, even at the lower pressure, cells displayed a robust uniform response, as measured by a change in $LimE_{\Delta coil}$ localization, that preceded any change in the position of the cell itself. Thus, these experiments clearly showed that 1) the global response does not depend on the movement of the cell, and 2) the global response precedes directed migration.

Alternative approaches of testing response to acute mechanical stimulation

Although the use of a flow-through chamber has clear advantages for the study of the response to acute mechanical stimulation, we also validated two additional assays for testing the response of individual cells to acute mechanical stimulation. As shown in **Figure 4**, $LimE_{\Delta coil}$ rapidly and transiently re-localized from the cytosol to the cortex when cells were stimulated by bolus addition of buffer delivered either by a micropipette (**Figure 4A**) or manually using bulk buffer addition with a pipette (**Figure 4B**). It should be noted that a clear disadvantage of both of these assays is that the exact amount of shear force delivered to the cell is unknown, and cannot be easily varied. However, for situations where switching between mechanical and chemical stimuli is desired, bulk buffer addition offers a solid alternative to the stimulation in the flow device.

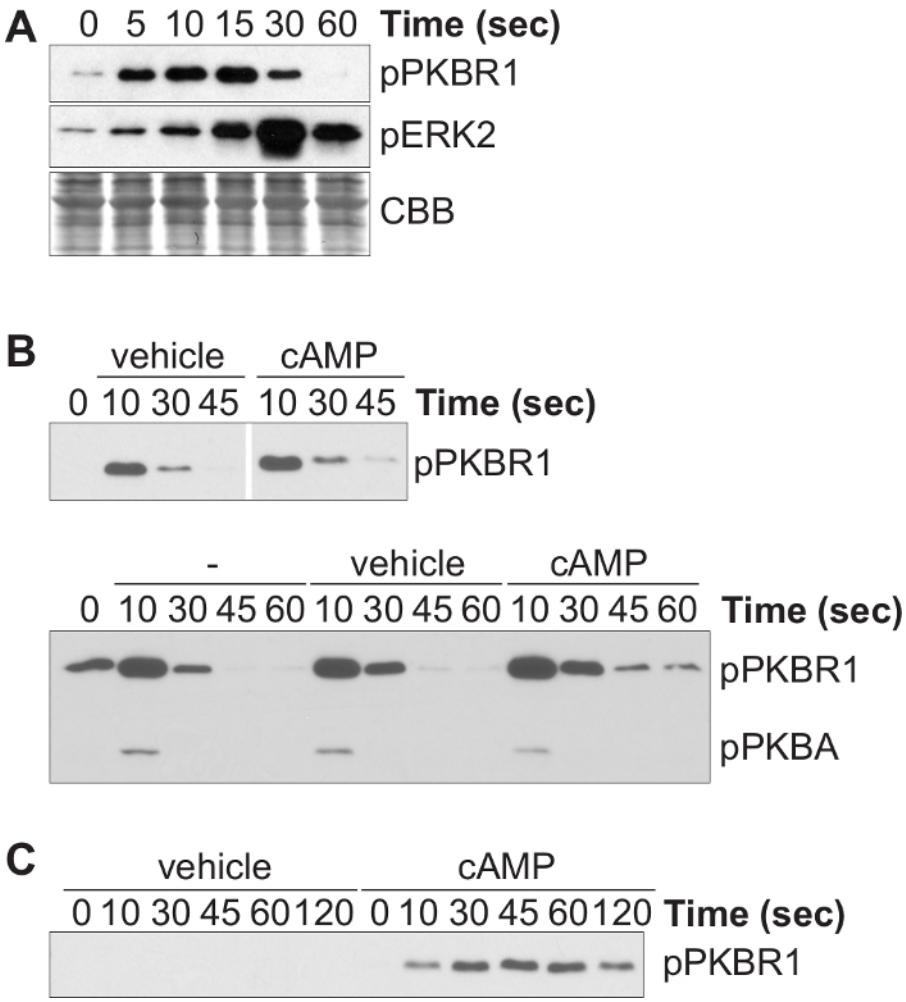

Figure 1: Representative results for biochemical assessment of the response of *D. discoideum* cells to acute mechanical or chemical stimulation. Aggregation-competent cells were exposed to mechanical or chemical stimuli, lysed at the indicated time points, and immunoblotted using antibodies that recognize phosphorylated PKBR1 or ERK2. (**A**) Adherent cells were stimulated on an orbital shaker for 5 s. The membrane was stained with Coomassie Brilliant Blue (CBB) to show equal protein loading. (**B**) Adherent cells were stimulated by manual movement of the plate in a cross-wise manner for approximately 5 s following chemical stimulation due to addition of 1 µM cAMP or buffer (vehicle), or without any chemical stimulation (-). The two immunoblots show the extent of variation in the basal activation of PKBR1 seen at time 0. Occasionally, PKBA activation could also be detected by this technique, as shown in the lower panel. (**C**) Suspension cells were stimulated with 1 µM cAMP or buffer (vehicle). Part (A) of this figure has been modified from Artemenko *et al*. (2016)[22]. Please click here to view a larger version of this figure.

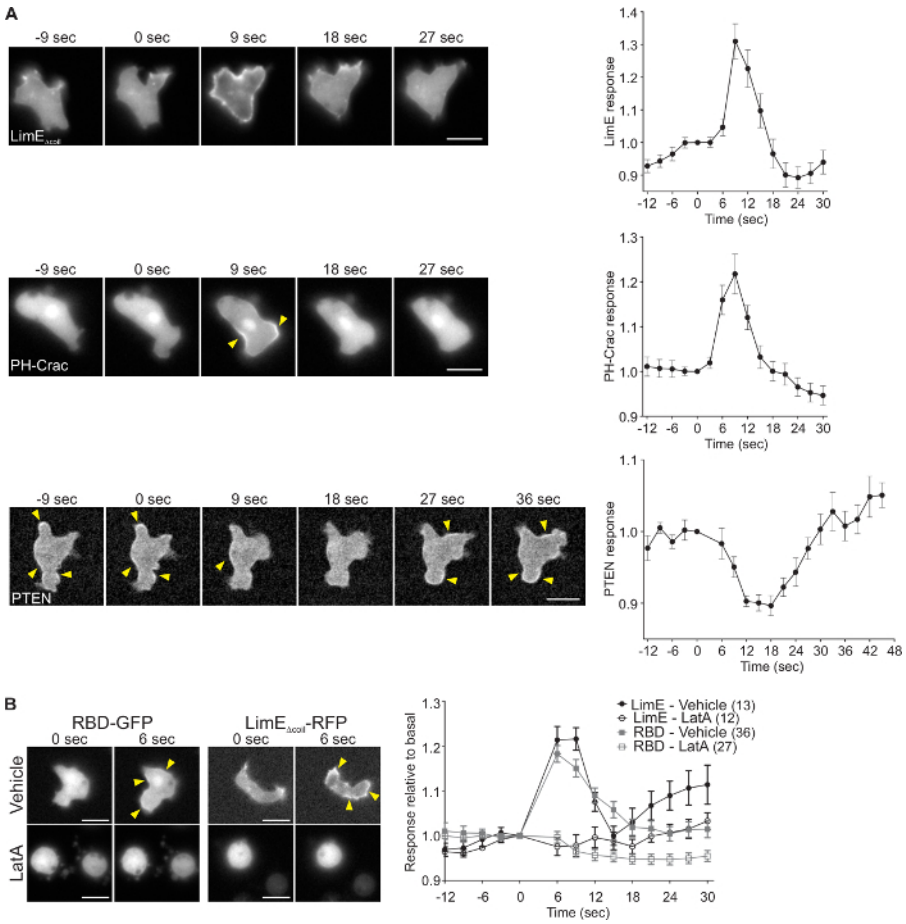

Figure 2: Representative results for acute mechanical stimulation and live imaging of single cells on the microscope. (**A**) Aggregation-competent *D. discoideum* cells expressing RFP-tagged $LimE_{\Delta coil}$, or GFP-tagged PH-Crac or PTEN were stimulated with unidirectional laminar flow at 15 dyn/cm^2 for 2 to 5 s. Images were collected every 3 s. Representative cells showing translocation of the biosensors in response to mechanical stimulation are shown. Accumulation of each biosensor at the cortex was quantified as the inverse of the mean cytoplasmic intensity normalized for time 0. The average response of 18 ($LimE_{\Delta coil}$), 20 (PH-Crac), or 6 (PTEN) cells is shown. Values are mean ± SE. (**B**) Vegetative cells expressing RFP-tagged $LimE_{\Delta coil}$-RFP and GFP-tagged RBD were treated with vehicle or 5 µM LatA for at least 15 min, and then stimulated with unidirectional laminar flow at 41 dyn/cm^2 for 2 s. Images were collected every 3 s. RBD-GFP and $LimE_{\Delta coil}$-RFP accumulation at the cortex was quantified as in (**A**). Values are mean ± SE. The number of cells analyzed is indicated beside each condition. Scale bar = 10 µm. This figure has been modified from Artemenko *et al.* (2016)[22]. Please click here to view a larger version of this figure.

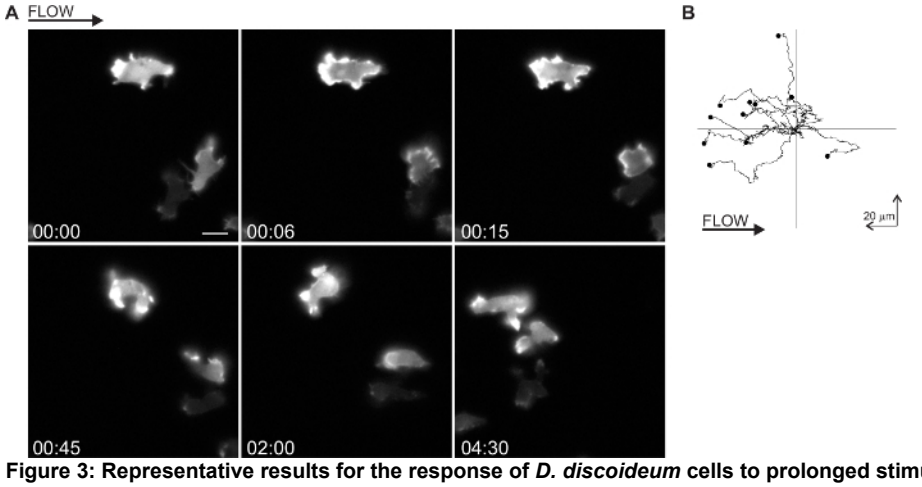

Figure 3: Representative results for the response of *D. discoideum* cells to prolonged stimulation with flow. (**A**) Vegetative cells expressing RFP-tagged $LimE_{\Delta coil}$ were exposed to continuous unidirectional laminar flow at 15 dyn/cm^2 and imaged every 3 s. Tracks of 11 cells are shown in (**B**). Scale bar = 10 µm. This figure has been modified from Artemenko *et al.* (2016)[22]. Please click here to view a larger version of this figure.

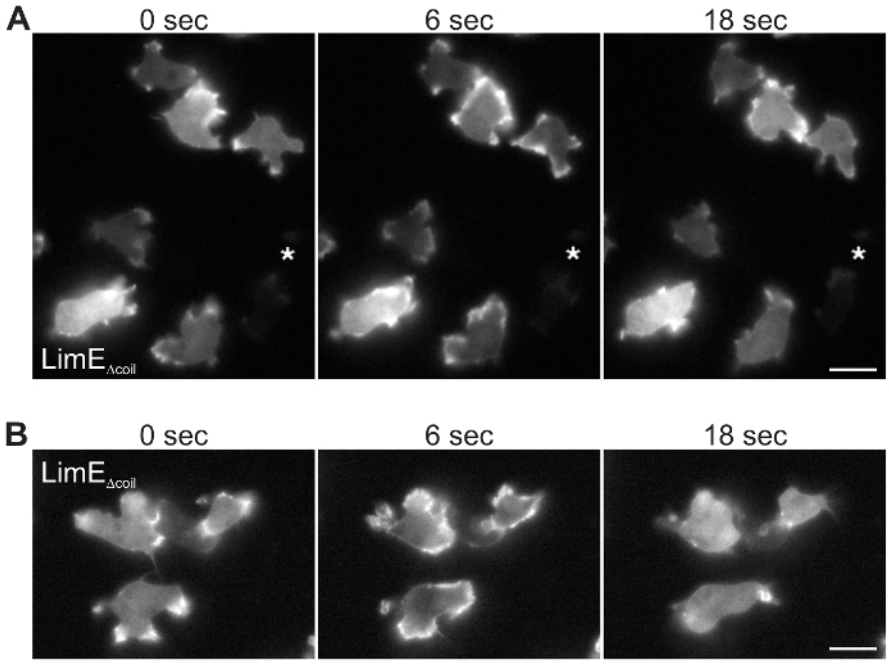

Figure 4: Representative results for alternative approaches to testing response to acute mechanical stimulation. (**A**) Vegetative *D. discoideum* cells expressing RFP-tagged LimE$_{\Delta coil}$ were exposed to a micropipette (*) basally releasing assay buffer at a slow rate. A brief increase in flow rate was achieved by rapid delivery of a bolus of assay buffer at time 0. Images were collected every 3 s. (**B**) Vegetative *D. discoideum* cells expressing RFP-tagged LimE$_{\Delta coil}$ plated as a small drop in a microscopy chamber were mechanically stimulated by addition of a large volume of buffer to the chamber. Images were collected every 3 s. Scale bar = 10 µm. Part (**A**) of this figure has been modified from Artemenko *et al.* (2016)[22]. Please click here to view a larger version of this figure.

Supplemental Movie 1: Acute mechanical stimulation triggers rapid and transient translocation of LimE$_{\Delta coil}$-RFP or PH-Crac-GFP from the cytosol to the cortex in aggregation-competent *D. discoideum* cells expressing these biosensors. Unidirectional laminar flow was turned on for 5 s at time 0 and cells were imaged at 3 s intervals. Playback speed is 5 frames/s. Two examples for each cell line are shown. Scale bar = 10 µm. This movie corresponds to **Figure 2A** and has been modified from Artemenko *et al.* (2016)[22]. Please click here to download this file.

Supplemental Movie 2: Continuous mechanical stimulation triggers rapid and transient translocation of LimE$_{\Delta coil}$-RFP from the cytosol to the cortex prior to migration of cells against the direction of the flow. Vegetative cells expressing LimE$_{\Delta coil}$-RFP were exposed to continuous shear stress at 15 dyn/cm^2 starting at time 0 and imaged at 3 s intervals. Playback speed is 10 frames/s. Scale bar = 10 µm. This movie corresponds to **Figure 3** and has been previously published in Artemenko *et al.* (2016)[22]. Please click here to download this file.

Discussion

The methods described here offer a convenient way to assess both population and individual cell behavior in response to shear flow. Importantly, while previous studies analyzed localization of leading and lagging edge markers during migration, the current approach allows investigation of immediate effects by applying the shear flow acutely. Using this method we demonstrated that, in fact, the initial response of cells to shear flow does not require cell migration. Instead, the rapid and transient response to the initial shear flow stimulus precedes the directional migration seen with prolonged exposure to shear flow, similarly to the initial global response seen with a chemoattractant gradient.

The biochemical and microscopic approaches yield complementary data even though each method has distinct advantages and disadvantages. Stimulation followed by cell lysis and immunoblotting of PKBs, KrsB, and ERK, for example, analyzes an entire population of cells and thus averages cell-to-cell variation, unlike an assay that examines individual cell behavior. However, population-based assays tend to mask subtle changes in cell behavior that can be easily observed by analyzing individual cells. Additionally, the biochemical assay described here is only effective with aggregation-competent cells, for reasons that will be discussed later. In contrast, the microscopy-based assay of the relocalization of RBD, RalGDS, PH-Crac, LimE$_{\Delta coil}$, and PTEN, for example, between the cortex and cytosol is effective for both vegetative and aggregation-competent cells, and thus allows for observations that are broadly applicable to cells with varying degrees of polarization. What makes the two approaches complementary is the fact that they examine different sets of available leading/lagging edge markers, thus expanding the coverage of the signal transduction and actin cytoskeleton networks tested with the shear flow assay.

It remains unclear why vegetative cells, which respond very robustly in microscopy-based assays, do not respond to stimulation followed by cell lysis and immunoblotting. One possibility is that the amount of shear force applied to cells when the plate is rotated does not match the force experienced by the cells in a flow chamber. Developed cells, in contrast, might have a lower threshold for activation, and, thus, respond under either condition. It is unlikely that the biosensors whose activity is measured by the biochemical assay are not expressed or are not activated in vegetative cells, since stimulation of vegetative cells with folic acid leads to robust activation of both PKBR1/PKBA and ERK2 (data not shown). Moreover, vegetative cells show clear recruitment of PH-Crac, which reflects PIP3 accumulation, in the microscopy-based assay. Since PKBA

recruitment also depends on PIP3, it seems unlikely that PKBA would not respond to acute mechanical stimulation, unless the conditions in the biochemical assay are not optimal as mentioned above. This remains an area of active investigation.

The biochemical analysis of chemotaxis regulators required the presence of caffeine throughout the experiment. Originally caffeine was added to prevent cell to cell signaling due to secretion of cAMP. However, it appears that caffeine has another role besides inhibition of adenylyl cyclase (ACA) since ACA-null cells still required caffeine for phosphorylation of PKBR1 in response to acute mechanical stimulation. Caffeine has been previously shown to block TorC2 activity[26]. It is possible that inhibition of TorC2 blocked some basal motility of the cells, and thus lowered the basal activation of PKBR1 and other components of signal transduction and actin cytoskeleton networks. Low basal activity was imperative for observing the response to shear flow. In fact, when cells were collected at earlier time points during development, they displayed increased basal activity and a reduced shear-flow induced response. Interestingly, it is known that vegetative cells have proportionally more PKBA activity and less PKBR1 compared to developed cells[31]. It is possible that the basal state is regulated somewhat differently in vegetative and aggregation-competent cells, further contributing to the lack of a response of vegetative cells in the biochemical assay. Curiously, when cells were collected at later points of development, they also had a reduced response, likely due to the strong influence of polarity on the localization and activation of various chemotaxis regulators examined in this assay.

Stimulation of cells in a microscopy chamber revealed that both the strength and the duration of stimulus contribute to maximal response[22]. In other words, a maximal response can be achieved even with a low shear force if it is applied for a prolonged period of time (10 s versus 2 s). This observation explains why cells have an initial global response when they are first exposed to a continuous, but weak, stimulus that leads to shear flow-induced migration. With the current apparatus, we were unable to generate low enough shear flow to investigate the lower end of the range.

Perhaps, the most important implication of the studies conducted using acute mechanical stimulation is that both mechanical and chemical stimuli appear to feed into common signal transduction and actin cytoskeleton networks. Although we showed that the two stimuli integrate using bulk buffer addition for the mechanical stimulation, future studies will aim to develop a system where chemoattractant can be delivered rapidly in the flow-through channel, which would be a much more versatile and powerful way to assess integration of mechanical and chemical stimuli. Unfortunately, chemoattractant addition using the current two-line setup is associated with a second shear flow stimulus because the chemoattractant has to be delivered in the presence of flow. A viable alternative may be laser-stimulated release of a chemoattractant from a caged precursor as was successfully shown for cAMP by Westendorf *et al*.[32]. In the future, it would also be interesting to examine integration of other stimuli, for example, shear flow and variable electric fields, or shear flow and variable substrate stiffness. The latter example is interesting because it involves two types of mechanical stimulation, although the initial signal reception might differ between them. Confirming that all stimuli that induce directed migration share the same signal transduction network and vary only in how the stimulus is perceived will explain how cells can navigate in complex environments. Finally, we already demonstrated that acute stimulation with shear flow leads to spreading of human neutrophil-like cells[22]. Future work will further examine localization and activation of various components of the signal transduction and actin cytoskeleton networks with mechanical stimuli in mammalian cells.

Disclosures

The authors have nothing to disclose.

Acknowledgements

This work was supported by National Institutes of Health grant R35 GM118177 to PND.

References

1. Swaney, K. F., Huang, C. H., & Devreotes, P. N. Eukaryotic chemotaxis: a network of signaling pathways controls motility, directional sensing, and polarity. *Annu Rev Biophys.* **39** 265-289 (2010).
2. Sasaki, A. T., Chun, C., Takeda, K., & Firtel, R. A. Localized Ras signaling at the leading edge regulates PI3K, cell polarity, and directional cell movement. *J Cell Biol.* **167** (3), 505-518 (2004).
3. Jeon, T. J., Lee, D.-J., Merlot, S., Weeks, G., & Firtel, R. A. Rap1 controls cell adhesion and cell motility through the regulation of myosin II. *J Cell Biol.* **176** (7), 1021-1033 (2007).
4. Parent, C. A., Blacklock, B. J., Froehlich, W. M., Murphy, D. B., & Devreotes, P. N. G Protein Signaling Events Are Activated at the Leading Edge of Chemotactic Cells. *Cell.* **95** (1), 81-91 (1998).
5. Funamoto, S., Meili, R., Lee, S., Parry, L., & Firtel, R. A. Spatial and Temporal Regulation of 3-Phosphoinositides by PI 3-Kinase and PTEN Mediates Chemotaxis. *Cell.* **109** (5), 611-623 (2002).
6. Iijima, M., & Devreotes, P. Tumor Suppressor PTEN Mediates Sensing of Chemoattractant Gradients. *Cell.* **109** (5), 599-610 (2002).
7. Lim, C. J., Spiegelman, G. B., & Weeks, G. RasC is required for optimal activation of adenylyl cyclase and Akt/PKB during aggregation. *EMBO J.* **20** (16), 4490-4499 (2001).
8. Kamimura, Y. *et al.* PIP3-Independent Activation of TorC2 and PKB at the Cell's Leading Edge Mediates Chemotaxis. *Curr Biol.* **18** (14), 1034-1043 (2008).
9. Kortholt, A., King, J. S., Keizer-Gunnink, I., Harwood, A. J., & Van Haastert, P. J. M. Phospholipase C Regulation of Phosphatidylinositol 3,4,5-trisphosphate-mediated Chemotaxis. *Molecular Biology of the Cell.* **18** (12), 4772-4779 (2007).
10. Huang, C. H., Tang, M., Shi, C., Iglesias, P. A., & Devreotes, P. N. An excitable signal integrator couples to an idling cytoskeletal oscillator to drive cell migration. *Nat Cell Biol.* **15** (11), 1307-1316 (2013).
11. Nishikawa, M., Hörning, M., Ueda, M., & Shibata, T. Excitable Signal Transduction Induces Both Spontaneous and Directional Cell Asymmetries in the Phosphatidylinositol Lipid Signaling System for Eukaryotic Chemotaxis. *Biophys J.* **106** (3), 723-734 (2014).

12. Gerisch, G. *et al.* Mobile Actin Clusters and Traveling Waves in Cells Recovering from Actin Depolymerization. *Biophys J.* **87** (5), 3493-3503 (2004).
13. Gerisch, G., Ecke, M., Wischnewski, D., & Schroth-Diez, B. Different modes of state transitions determine pattern in the Phosphatidylinositide-Actin system. *BMC Cell Biol.* **12** (1), 42 (2011).
14. Bretschneider, T. *et al.* The Three-Dimensional Dynamics of Actin Waves, a Model of Cytoskeletal Self-Organization. *Biophys J.* **96** (7), 2888-2900 (2009).
15. Weiner, O. D., Marganski, W. A., Wu, L. F., Altschuler, S. J., & Kirschner, M. W. An Actin-Based Wave Generator Organizes Cell Motility. *PLOS Biol.* **5** (9), e221 (2007).
16. Devreotes, P., & Horwitz, A. R. Signaling Networks that Regulate Cell Migration. *Cold Spring Harbor Perspectives in Biology.* **7** (8) (2015).
17. Artemenko, Y., Lampert, T. J., & Devreotes, P. N. Moving towards a paradigm: common mechanisms of chemotactic signaling in Dictyostelium and mammalian leukocytes. *Cell Mol Life Sci.* **71** (19), 3711-3747 (2014).
18. Dalous, J. *et al.* Reversal of cell polarity and actin-myosin cytoskeleton reorganization under mechanical and chemical stimulation. *Biophys J.* **94** (3), 1063-1074 (2008).
19. Sato, M. J. *et al.* Switching direction in electric-signal-induced cell migration by cyclic guanosine monophosphate and phosphatidylinositol signaling. *Proc Natl Acad Sci U S A.* **106** (16), 6667-6672 (2009).
20. Zhao, M., Pu, J., Forrester, J. V., & McCaig, C. D. Membrane lipids, EGF receptors, and intracellular signals colocalize and are polarized in epithelial cells moving directionally in a physiological electric field. *Faseb j.* **16** (8), 857-859 (2002).
21. Decave, E. *et al.* Shear flow-induced motility of Dictyostelium discoideum cells on solid substrate. *J Cell Sci.* **116** (Pt 21), 4331-4343 (2003).
22. Artemenko, Y., Axiotakis, L., Borleis, J., Iglesias, P. A., & Devreotes, P. N. Chemical and mechanical stimuli act on common signal transduction and cytoskeletal networks. *Proc Natl Acad Sci U S A.* **113** (47), E7500-E7509 (2016).
23. Artemenko, Y., Swaney, K. F., & Devreotes, P. N. Assessment of development and chemotaxis in Dictyostelium discoideum mutants. *Methods Mol Biol.* **769** 287-309 (2011).
24. Gaudet, P., Pilcher, K. E., Fey, P., & Chisholm, R. L. Transformation of Dictyostelium discoideum with plasmid DNA. *Nat. Protocols.* **2** (6), 1317-1324 (2007).
25. Veltman, D. M., Lemieux, M. G., Knecht, D. A., & Insall, R. H. PIP3-dependent macropinocytosis is incompatible with chemotaxis. *J Cell Biol.* **204** (4), 497-505 (2014).
26. Cai, H., Huang, C. H., Devreotes, P. N., & Iijima, M. Analysis of chemotaxis in Dictyostelium. *Methods Mol Biol.* **757** 451-468 (2012).
27. Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat Meth.* **9** (7), 676-682, (2012).
28. Brenner, M., & Thoms, S. D. Caffeine blocks activation of cyclic AMP synthesis in Dictyostelium discoideum. *Dev Biol.* **101** (1), 136-146 (1984).
29. Bretschneider, T. *et al.* Dynamic Actin Patterns and Arp2/3 Assembly at the Substrate-Attached Surface of Motile Cells. *Curr Biol.* **14** (1), 1-10 (2004).
30. Swaney, K. F., Borleis, J., Iglesias, P. A., & Devreotes, P. N. Novel protein Callipygian defines the back of migrating cells. *Proc Natl Acad Sci U S A.* **112** (29), E3845-3854 (2015).
31. Meili, R., Ellsworth, C., & Firtel, R. A. A novel Akt/PKB-related kinase is essential for morphogenesis in Dictyostelium. *Curr Biol.* **10** (12), 708-717 (2000).
32. Westendorf, C. *et al.* Actin cytoskeleton of chemotactic amoebae operates close to the onset of oscillations. *Proc Natl Acad Sci U S A.* **110** (10), 3853-3858 (2013).

