Chapter 12

Evaluating the Activity of Ras/Rap GTPases in *Dictyostelium*

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Abstract

Ras and Rap small GTPases of the Ras superfamily act as molecular switches to control diverse cellular processes as part of different signaling pathways. *Dictyostelium* expresses several Ras and Rap proteins, and their study has and continues to greatly contribute to our understanding of their role in eukaryote biology. To study the activity of Ras and Rap proteins in *Dictyostelium*, several assays based on their interaction with the Ras binding domain of known eukaryotic Ras/Rap effectors have been developed and proved extremely useful to study their regulation and cellular roles. Here, we describe methods to assess Ras/Rap activity biochemically using a pull-down assay and through live-cell imaging using fluorescent reporters.

Key words *Dictyostelium discoideum*, Ras, Rap1, Activity assays, Ras binding domain, Pull-down, Imaging

1 Introduction

Ras and Ras-related protein (Rap) GTPases play key roles in numerous biological processes that are studied in *Dictyostelium*, including cell proliferation, chemotaxis, cell adhesion, and phagocytosis [1]. In *Dictyostelium*, there are 11 Ras and 3 Rap proteins, but only 7 of them have been studied thus far: RasB, RasC, RasD, RasG, RasS, RapA (Rap1), and, more recently, RapC [1–4]. RasB, RasC, RasD, and RasG are most closely related to each other and to human Ras proteins whereas RasS is more divergent; and Rap1 was found to be the homologue of human Rap1 whereas RapC is more divergent.

Ras/Rap GTPases act as molecular switches, cycling between an inactive GDP-bound form and an active GTP-bound form. To study the activity of Ras/Rap in cells, methods using the Ras binding domain (RBD) of known Ras/Rap effectors have been developed, which allow detecting GTP-bound Ras/Rap proteins both biochemically by pull-down (Fig. 1a) and in live cells with fluorescent reporters and imaging approaches (Fig. 1b), and these

Alan R. Kimmel (ed.), *Dictyostelium discoideum: Methods and Protocols*, Methods in Molecular Biology, vol. 2814,
https://doi.org/10.1007/978-1-0716-3894-1_12,
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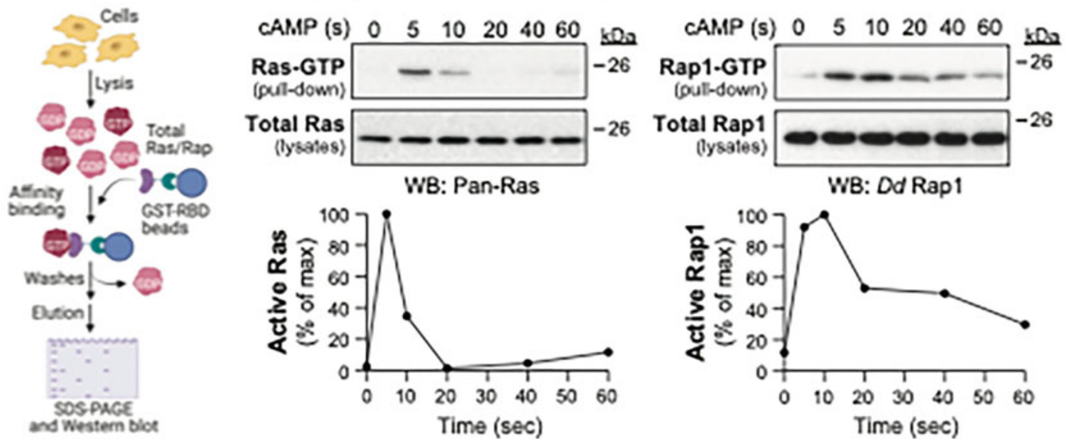

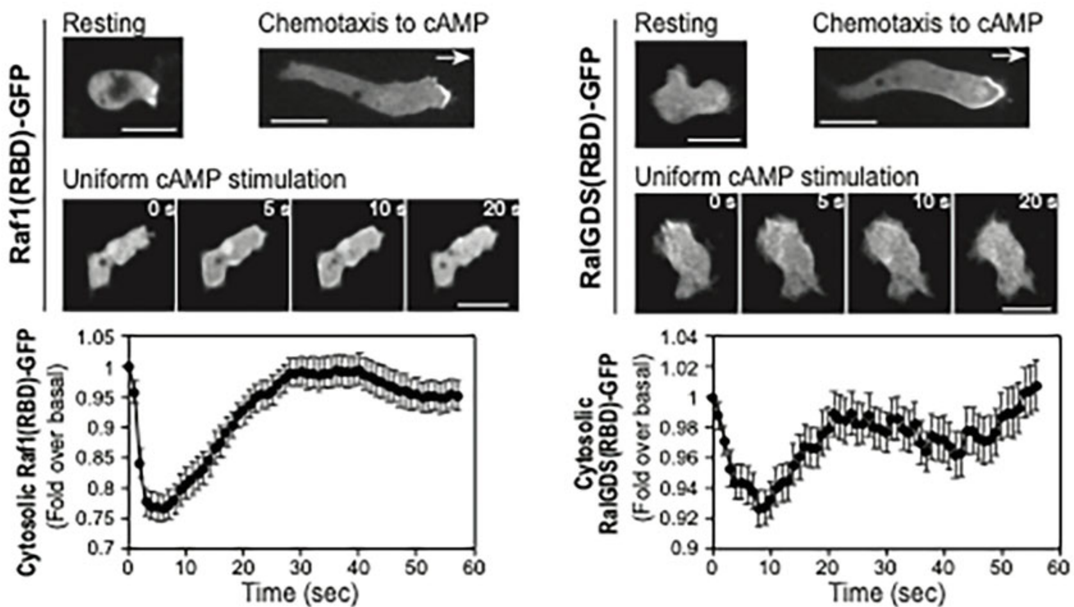

Fig. 1 Ras/Rap activity assays. Examples of data obtained with 5.5 h developed AX3 cells are shown. (**a**) The Ras/Rap activity pull-down assay. An outline of the assay is depicted on the left. Cells were stimulated with cAMP for the indicated time before lysing the cells and purifying the GTP-bound Ras or Rap proteins using GST-Raf1(RBD) for Ras and GST-RalGDS(RBD) for Rap1, followed by their analysis by Western blot using a Pan-Ras antibody or *Dictyostelium* (Dd) Rap1-specific antibody. The graphs illustrate the relative cAMP-induced activation of the Ras and Rap1 proteins as quantified by densitometry, normalized to the total Ras and Rap1, and expressed as percentage of the maximal response for each. (**b**) The Ras/Rap activity imaging assay. The Raf1(RBD)-GFP and RalGDS(RBD)-GFP were used as reporters of Ras and Rap1 activity, respectively. Images show localization of the reporters and, thereby, active Ras and Rap1, in resting/randomly moving cells, cells chemotaxing to cAMP in a pipette chemotaxis assay, and upon the uniform stimulation of cells with cAMP

have been successfully adapted for use in *Dictyostelium* [5, 6]. Commonly, the RBD of the human Rap1 effector RalGDS is used to assay *Dictyostelium* Rap1 activity [6]; the RBD of human Ras effector Raf1 is used to assay RasB, RasD, and RasG activity; and the RBD of the yeast Ras effector Byr2 is used to assay RasC activity [5, 7]. Of note, RasC has a slightly different effector domain sequence, and because of that, it does not interact effectively with Raf1-RBD [5]. On the other hand, Byr2-RBD interacts with RasC as well as the other Ras proteins and Rap1, but because its production in bacteria produces much lower yields than Raf1-RBD and RalGDS-RBD, those are still preferentially used with the other Ras/Rap proteins. Moreover, while Byr2-RBD is a great tool in Ras/Rap activity pull-down assays, especially for RasC, it does not work well as a fluorescent reporter in live cells due to its too high affinity to the GTP-bound Ras/Rap proteins, which leads to Ras/-Rap function inhibition. Here, we describe the biochemical and imaging methods to spatiotemporally detect and quantify Ras/Rap activity in *Dictyostelium* (Fig. 1).

2 Materials

Prepare all solutions with ultrapure deionized water (ddH_2O) and sterilize the ones that are used with cells.

2.1 Ras/Rap Activity Pull-Down Assay

2.1.1 GST-RBD Expression and Purification

1. BL21 DE3-competent bacteria.
2. GST-Byr2(RBD), GST-Raf1(RBD), and/or GST-RalGDS (RBD) bacterial expression plasmids, depending on the Ras/Rap proteins to be assayed (*see* **Note 1**).
3. LB media and LB agar plates with appropriate selection antibiotic.
4. Incubator shaker capable of maintaining temperatures of 16 and 37 °C.
5. GSH agarose beads and Sephadex G-10 beads.
6. 6× Protein sample buffer: 0.375 mM Tris-HCl pH 6.8, 30% glycerol, 6% SDS, 600 mM DTT, and 6% bromophenol blue, in ddH_2O. Aliquot and freeze at −20 °C.
7. 1 M IPTG: dissolve in ddH_2O, aliquot, and store at −20 °C.

Fig. 1 (continued) for the indicated time. The graphs show the relative fluorescence of the RBD-GFP reporter in the cytosol in cAMP-stimulated cells, as mean ± S.E.M of more than 20 cells from three different experiments, expressed as fold over the basal level. The fluorescence intensity in the cytosol is inversely proportional to that at the plasma membrane and, as such, inversely reflects Ras and Rap1 activation at the plasma membrane. The data shown here is provided as an example and was originally published in Scavello et al. [11]

8. Phosphate-buffered saline (PBS): 116 mM NaCl, 13.2 mM Na_2HPO_4, and 1.47 mM KH_2PO_4, in ddH_2O. Adjust pH to 7.4 with HCl. Store at 4 °C. Before use, supplement PBS with a bacteria-specific protease inhibitor cocktail.
9. 50% glycerol/PBS: mix 1 part glycerol with 1 part PBS. Store at 4 °C.

2.1.2 Pull-Down of Active Ras/Rap

1. Tabletop centrifuge and microcentrifuge.
2. Gyratory shaker for flasks.
3. Rotator or gyratory rocker for sample tubes.
4. 100 mg/mL BSA: dissolve in ddH_2O, aliquot, and store at −20 °C.
5. 2× Ras/Rap lysis buffer: 100 mM Tris pH 7.5, 300 mM NaCl, 50 mM $MgCl_2$, 20% glycerol, and 1% NP-40 or IGEPAL CA-630, in ddH_2O. Store at 4 °C. Before the assay, prepare a working solution by adding 2 mM DTT, 2 mM Orthovanadate, 4 μg/mL aprotinin, and 4 μg/mL leupeptin (*see* **Note 2**).
6. 12 mM Na/K phosphate buffer: 2.5 mM Na_2HPO_4, 9.5 mM KH_2PO_4, and pH 6.1. To prepare a 1 L10× solution, dissolve 3.4 g Na_2HPO_4 and 13 g KH_2PO_4 in ddH_2O. Autoclave to sterilize and store at 22 °C (*see* **Note 3**).
7. 30 mM cAMP stock: dissolve cAMP-Na salt monohydrate in 12 mM Na/K phosphate buffer. Aliquot and store at −20 °C.
8. 10 μM cAMP: dilute from 30 mM cAMP stock with Na/K phosphate buffer.

2.1.3 Analysis of Ras/Rap Activity by Western Blot

1. Tris-buffered saline (TBS): 50 mM Tris base, 150 mM NaCl, and 2.7 mM KCl, in ddH_2O. Adjust pH to 7.4 with HCL. Store at 4 °C.
2. TBS-Tween (TBST): Dilute Tween-20 to 0.1% in TBS.
3. Appropriate antibody: Pan-Ras, *Dictyostelium* Ras- or Rap-specific, or anti-epitope antibodies depending on goal of assay (*see* **Note 4**).
4. Software to perform densitometry analyses (e.g., ImageJ).

2.2 In Vivo Ras/Rap Activity Imaging Assay

2.2.1 Preparation of Cells

1. *Dictyostelium* expression plasmids for Raf1(RBD)-GFP (for detecting active RasB, RasD, and RasG) or RalGDS(RBD)-GFP (for detecting active Rap1) (*see* **Note 5**).
2. Coverslip-bottom dishes: Can be purchased or made from regular dishes. To make them, use a hole puncher to make holes of ~1 cm in diameter in the bottom and lid of 35 mM dishes. The bottoms can be used for imaging resting cells or for random cell motility and uniform stimulation assays, and the

lids (making shallower "dishes") are used for imaging cells in the micropipette chemotaxis assay because they allow more room to properly position the micropipette. Use vacuum grease or epoxy to secure 22 × 22 mM glass coverslips #1.5 over the holes, from the outside (*see* **Note 6**).

2.2.2 Live Cell Imaging

1. 150 μM cAMP: dilute from 30 mM cAMP stock in 12 mM Na/K phosphate buffer.

1. Spinning disk confocal imaging system equipped with a 488 laser, DIC capability, CCD camera, and 20×, 40×, and, nice but not necessary, 63× objectives.
2. Eppendorf TransferMan® micromanipulator.
3. Eppendorf Microloader™ tips and Femtotip® micropipettes.

2.2.3 Image Analysis

1. Confocal images analysis software (e.g., ImageJ/FIJI).

3 Methods

Ras/Rap activity can be measured in *Dictyostelium* cells at any stage of their life cycle and in many different conditions. We describe a protocol for assaying Ras/Rap activities in response to cAMP in 5.5 h. developed, aggregation-competent cells that are highly responsive to cAMP, but the method can be easily applied to other cellular contexts and conditions. Cells should be prepared using standard *Dictyostelium* cell culture and development methods [8].

3.1 Ras/Rap Activity Pull-Down Assay

3.1.1 GST-RBD Expression and Purification

1. Transform the desired GST-RBD-encoding plasmid into competent bacteria using standard procedures.
2. Pick a colony and grow in 10 mL LB overnight at 37 °C with shaking at 300 rpm in an incubator shaker.
3. Transfer the overnight culture to 1 L of LB in a 2.5 L flask (*see* **Note 7**).
4. Grow bacteria at 37 °C with shaking at 300 rpm in an incubator shaker until the culture reaches an O.D. of 0.6–0.8.
5. Induce protein expression by adding 1 mM IPTG and incubate at 16 °C with shaking at 300 rpm in an incubator shaker for 16 h.
6. Collect the bacteria and pellet by centrifugation at 5000 × *g* for 20 min at 4 °C (*see* **Note 8**).
7. Resuspend cells in 20 mL cold PBS containing protease inhibitors.
8. Sonicate on ice, at 50% amplitude and duty cycle 5, 10 × 1 min with 2 min cooldown between each sonication.

9. Add NP-40 (or IGEPAL CA-630) to 1% final concentration, mixing for ~10 min on ice until complete dissolution.
10. Transfer the lysates to appropriate centrifuge tubes and clarify by centrifugation at 20,000 × *g* for 30 min at 4 °C.
11. Transfer the clarified lysates to a new tube with 1 mL of GSH Sepharose beads and incubate for at least 1 h at 4 °C with agitation on rotator or gyratory rocker.
12. Pellet the beads by centrifugation at 1000 × *g* for 1 min and wash five times with cold PBS.
13. Pellet the beads, remove PBS, and add 250 μL of 50% glycerol/PBS.
14. Take a 20 μL aliquot, mix with 4 μL 6× protein sample buffer, and quantify approximately by SDS-PAGE and Coomassie staining against 1, 2, 5, and 10 μg BSA standards using standard procedures.
15. Dilute the GST-RBD beads to 0.5 μg/μL with 50:50 hydrated Sephadex G-10 beads in 50% glycerol/PBS. Store at −20 °C.

3.1.2 Pull-Down of Active Ras/Rap

To measure the total Ras activity or the activity of overexpressed Ras/Rap proteins, we suggest using 1×10^8 cells, and to measure the activity of a single Ras/Rap protein expressed at endogenous or low levels, we suggest using 5×10^8 cells.

1. Collect for 5.5 h developed cells by centrifugation at 500 × *g* for 5 min at 4 °C.
2. Wash the cells twice with cold 12 mM Na/K phosphate buffer.
3. Resuspend the cells in 5 mL of 12 mM Na/K phosphate buffer, and incubate for 30 min at 22 °C with shaking at ~140 rpm on a gyratory shaker (*see* **Note 9**).
4. Transfer one 450 μL aliquot of cells to the "0" time point tube containing 500 μL 2× lysis buffer +50 μL 12 mM Na/K phosphate buffer and keep on ice.
5. Stimulate the remaining cells with 500 μL of 10 μM cAMP (1 μM final), and collect 500 μL aliquots at 5, 10, 20, 40, and 60 s, mixing the cells and changing the pipette tip between each sample, stopping the stimulation by transferring the aliquots to the corresponding pre-prepared tubes containing 500 μL 2× lysis buffer on ice (*see* **Note 10**).
6. Vortex the samples thoroughly and incubate on ice for 10 min, vortexing every 2 min.
7. Clarify the lysates by centrifugation at 20,000 × *g* for 10 min at 4 °C.
8. Transfer the supernatants to new tubes on ice and vortex thoroughly.

9. Collect 20 μL samples of the total cell lysates and mix with 20 μL 2× protein sample buffer (diluted from 6× protein sample buffer with ddH_2O) for total Ras Western blot. Store at −20 °C.
10. Add 20 μL of 100 mg/mL BSA (2 mg/mL final) and 20 μL of 0.5 μg/μL GST-RBD beads (10 μg final) to each lysate sample and incubate for 30 min–2 h at 4 °C with agitation on rotator or gyratory rocker (*see* **Note 11**).
11. Pellet the beads at 1000 × *g* for 1 min and wash three times with 1× lysis buffer (diluted from 2× lysis buffer with ddH_2O).
12. Carefully remove all lysis buffer and elute the Ras/Rap proteins from the beads by adding 30 μL of 2× protein sample buffer containing 100 mM DTT. Mix well and incubate at 65 °C for 15 min.
13. Vortex thoroughly and centrifuge at 1000 × *g* 1 min to bring down condensation, and then vortex and pellet beads again.
14. Carefully transfer the supernatants containing eluted Ras/Rap proteins to new tubes, without transferring any beads. Use immediately for Western blot analysis or store at −20 °C until needed.

3.1.3 Analysis of Ras/Rap Activity by Western Blot

1. Denature all proteins by incubating the samples at 95 °C for 10 min.
2. Centrifuge the heated samples to bring down condensation, and vortex to ensure sample homogeneity before loading and separating the proteins on 12% SDS-PAGE gels (*see* **Note 12**).
3. Transfer the proteins to a nitrocellulose membrane, perform the Western blot according to the appropriate antibody's manufacturer protocol, and reveal by chemiluminescence or fluorescence using standard procedures (*see* **Note 13**).
4. Save the Western blot images as grayscale JPEG files and open in an appropriate software for densitometric analyses (e.g., ImageJ).
5. If using ImageJ, set the measurement criteria to "Grey Mean Value" only and use the rectangle tool to draw a frame around the largest band to be measured, and then drag the frame to other bands and adjust the thickness accordingly to make sure that it can be used for all bands.
6. Using the same frame for all the Ras/Rap protein bands on the Western blot, measure the mean gray value (by clicking "Measure" under "Analyze" menu) for each band, one at a time. The first measurement will open a measurement window where all of them will be displayed in order to be taken.

7. Using the same frame, also take background measurements. If the background is very uniform, only one measurement can be taken, but if there are differences in background over the Western blot, take one background measurement next to each band.
8. Transfer the measurements to an Excel spreadsheet for analyses.
9. First, invert the pixel density for all data recorded by ImageJ (x) by subtracting them from 255 ($255 - x$), and then subtract the inverted background from all inverted band values.
10. Divide each GTP-Ras/Rap value by their corresponding total Ras/Rap loading control.
11. To facilitate comparison between separate experiments, because it is extremely challenging to have equivalent exposures of the GTP-Ras/Rap and total Ras/Rap Western blots between experiments, we suggest expressing the data as percent of the strongest response in the control condition (wild-type cells, no drug treatment, etc.).

3.2 In Vivo Ras/Rap Activity Imaging Assay

3.2.1 Preparation of Cells and Microscope

1. Generate *Dictyostelium* cells transformed with the appropriate RBD-GFP expressing plasmid and develop the cells for 5.5 h using standard procedures.
2. Transfer the cells to coverslip-bottom dishes containing 2 mL 12 mM Na/K phosphate buffer, at ~1×10^6 cells for random motility and uniform stimulation assays in a custom-made dish bottom with coverslip and at ~2.5×10^5 cells for a chemotaxis pipette assay in a custom-made dish lid with coverslip. Spread the cells evenly and let them settle and disaggregate for ~30 min (*see* **Note 14**).
3. Place a cell-containing coverslip-bottom dish on the stage of a spinning disk confocal imaging system and focus on cells that are on the coverslip using a 20× objective.
4. Find an area with multiple cells but that do not touch each other and that express the RBD-GFP reporter at low levels, which will allow for better contrast between the signal at the plasma membrane and the cytosol when the reporter is recruited to active Ras/Rap at the plasma membrane.
5. Set up the fluorescence imaging parameters, keeping the laser power as low as possible to obtain a clear image with a ~ 100 ms exposure, which will limit phototoxicity.

3.2.2 Imaging RBD-GFP in Resting, Randomly Moving, or Uniformly Stimulated Cells

1. For imaging resting and randomly moving cells, set up a time-lapse for capturing DIC (sometimes useful to see whole cells especially when the reporter expression is low) and fluorescence images at 6 s intervals for 30 min.

2. For imaging RBD-GFP in uniformly stimulated cells, set up a time-lapse for capturing fluorescence images at 1 s intervals for 60 s (or longer if desired). Start the acquisition and stimulate the cells on the third capture by adding 100 μL of 150 μM cAMP dropwise directly over the imaged cells (*see* **Note 15**).
3. To use as controls for photobleaching and background fluorescence in later analyses of the uniform stimulation experiment, also image unstimulated cells and cells not expressing any fluorescent reporter using the same time-lapse parameters.

3.2.3 Imaging RBD-GFP in Chemotaxing Cells Using a Micropipette Assay

1. Keeping the protective tip of a micropipette on, use a microloader tip to load a micropipette with 20 μL of 150 μM cAMP (*see* **Note 16**).
2. Set the micropipette holder of the micromanipulator to ~35°, carefully remove the micropipette's protective tip, and secure the micropipette on the holder according to the manufacturer's instructions.
3. Use a thin coverslip-bottom dish with cells (the ones made with the lids), and focus on the cells using a 20× objective in bright-field imaging or by looking through the eye piece directly.
4. Using the micromanipulator's control panel and joystick, position the tip of the micropipette in the middle of the objective, and start to slowly lower in the buffer. Focus up to find the tip of the micropipette in the field of view and gradually, very carefully, lower to ~10 μm above the cells (*see* **Note 17**).
5. Bring the micropipette a bit higher and then hit "home" on the micromanipulator's controller to take it out of the buffer. Change to the 40× objective (or 63× for higher resolution images), and find a good area to image, with several separated cells and where the micropipette can be positioned away from the cells so that they will not reach the tip before the end of the recording (*see* **Note 18**).
6. Hit "home" to bring the micropipette back into position and finely bring down to ~10 μm above the cells and place slightly away from cells, ideally at the edge of the field of view.
7. Set up a time-lapse for capturing DIC and fluorescence images at 6 s intervals for 10 min. To image the response upon introduction of the micropipette and gradient establishment, start the time-lapse before bringing the micropipette back.
8. Export images as RGB TIFF files or the entire recording as series movies for publication and/or presentations, and as 16-bit TIFF files (multi-page TIFF) for subsequent quantitative analyses.

3.2.4 Quantitative Analysis of Uniform cAMP Stimulations

1. Open the 16-bit TIFF files in an appropriate image analysis software such as ImageJ/FIJI.
2. For each condition and cell to be measured, select a cytosolic region omitting organelles with higher or lower fluorescence intensities, making sure the region is good for the entire time-lapse. The average value of all pixels within the selected cytosol region then serves as a measure of cytosolic fluorescence intensity at time t, $I_c(t)$ (*see* **Note 19**).
3. Use the measurements on cells not expressing any fluorescent reporter to determine the background fluorescence intensity at each time point, $I_b(t)$.
4. Average the fluorescence intensity measured in the first frames before cAMP was added to obtain the basal cytosolic fluorescence intensity, I_{c0} and I_{b0}.
5. Calculate the normalized fluorescence signals, $I_{c\ nor}(t)$ as follows: $I_{c,nor}(t) = [I_c(t) - I_b(t)]/(I_{c0} - I_{b0})$ (*see* **Note 20**).
6. To measure the photobleaching effect, first measure the fluorescence intensity of unstimulated RBD-GFP-expressing cells at each time point, $I_a(t)$, and normalize the time series by calculating $I_{a,nor}(t) = [I_a(t) - I_b(t)]/(I_{a0} - I_{b0})$.
7. Correct each fluorescence measurement of the RBD-GFP reporter for the photobleaching effect and determine the final cytosolic signal by calculating $I(t) = I_{c\ nor}(t)/I_{a\ nor}(t)$.
8. Plot the fluorescence intensity in the cytosol as a function of time to generate a graph showing the changes of cytosolic fluorescence intensity upon cAMP stimulation.

4 Notes

1. The RBD of yeast Byr2 can be used to pull down RasB, RasC, RasD, RasG, and Rap1; the RBD of human Raf1 can be used to pull down active RasB, RasD, and RasG (it does not interact with RasC); and the RBD of human RalGDS can be used to pull down Rap1. Of note, Raf1-RBD agarose and RalGDS-RBD agarose are commercially available, so they can be purchased instead of prepared as described in this protocol.
2. Excess 2× lysis buffer can be prepared to be diluted with Na/K phosphate buffer afterward and used as 1× lysis buffer for washing the beads.
3. An alternative buffer that can be used with *Dictyostelium* is the development buffer (DB; 5 mM Na_2HPO_4, 5 mM KH_2PO_4; 1 mM $CaCl_2$, 2 mM $MgCl_2$, pH 6.5).

4. Commercial Pan-Ras antibodies will detect many *Dictyostelium* Ras proteins, including RasB, RasC, RasD, and RasG. Alternatively, custom-made Ras/Rap-specific antibodies can be used to detect the activity of specific Ras/Rap proteins. Finally, if custom-made antibodies are unavailable and cells expressing epitope-tagged Ras/Rap proteins are used, epitope-specific antibodies are then used.
5. The Byr2(RBD) does not work as a reporter of Ras activity in live cells, presumably because its affinity for the Ras/Rap proteins is too strong and, consequently, inhibits their function. Therefore, it is important to note that none of the currently available fluorescent reporters of Ras activity report the activity of RasC. Also note that several versions of the fluorescent reporters exist, with the latest optimized ones by Arjan Kortholt [9, 10].
6. Vacuum grease should only be used when preparing dishes the same day of a planned experiment, to avoid it drying leading to buffer leaking out. Epoxy-secured coverslip-bottom dishes can be prepared in advance and can be used to culture cells before the experiment if needed.
7. For GST-Raf1(RBD) and GST-RalGDS(RBD), 1 L produces sufficient protein for multiple experiments. However, GST-Byr2(RBD) expression and purification from bacteria generate much lower yields, and we recommend using 5 L of culture for this one.
8. Bacteria can be frozen at −20 °C at this stage and then thawed directly into PBS when needed. Especially for GST-Byr2(RBD) that produces low protein yields, it is useful to grow larger volumes of culture, store aliquots of pelleted bacteria at −20 °C, and purify as needed.
9. During this incubation, prepare the tubes for the samples to be collected before (for basal activity level) and after cAMP stimulation: 0, 5, 10, 20, 40, and 60 s. Add 500 μL of 2× lysis buffer in each tube and place on ice. Add 50 μL of 12 mM Na/K phosphate buffer to the "0" time point tube, which is the equivalent of the cAMP solution that will be in the other samples. We find it helpful to use a rack to hold the tubes in place on ice.
10. Collecting the 5 and 10 s time points can be particularly challenging, especially with shaking constantly as much as possible. We hold the tube with the cells in one hand, mixing the cells after addition of cAMP and in between each collection, while using the other hand to collect and transfer samples to the tubes on ice. Between each time point, the tip is discarded, and another tip is rapidly mounted on the pipette from a racked tip box nearby. We strongly suggest changing tips, because for

optimal rapid stopping of the stimulation, it is best to deliver the samples *in* the lysis buffer so that some mixing occurs before all the time points are finished being collected, and if the tip is not changed, then there would be a risk of transferring some lysis buffer to the cells upon collection of the next time point sample. We also strongly suggest practicing doing this procedure to get used to it before doing a first experiment.

11. An incubation time of 30 min with the GST-RBD beads is enough and recommended when assaying total Ras activity or overexpressed Ras/Rap proteins, to limit binding of inactive Ras. However, when assaying the activity of single Ras/Rap protein expressed at endogenous or low levels, we have had better success incubating for longer times.
12. The total Ras/Rap proteins may give a considerably stronger signal, so we advise loading those on different gels than the active Ras/Rap samples. Endogenous Ras/Rap proteins migrate ~21 kDa.
13. For detecting single Ras/Rap1 proteins expressed at endogenous or low levels, we recommend using a super sensitive ECL reagent (e.g., SuperSignal™ West Femto Maximum Sensitivity Substrate) to facilitate detection of the pulled-down proteins, especially.
14. We suggest always preparing several dishes to have backups in case a dish leaks and to allow performing replicates in the stimulation assays.
15. To avoid disturbing the cells, which are not very adherent on the glass substrate, we suggest delivering the cAMP dropwise right above the buffer as quickly as possible. Because the cAMP cannot be mixed after addition, we found that using a higher cAMP concentration leads to more consistent results. Also note that the addition of cAMP can be done at later captures (instead of at the third one), the important thing is to be consistent to facilitate later analyses.
16. To avoid breaking the micropipette, keep the protective tip on during loading and until ready to mount on the micromanipulator. Also, it is important to make sure that there are no air bubbles in the micropipette, especially at the very tip as this will prevent cAMP from diffusing out. To get rid of air bubbles, one can give a good flick of the wrist while holding the micropipette, or centrifuge briefly in an appropriate microcentrifuge (which accommodates the long micropipette with the protective tip). Also of note, we suggest using 150 μM cAMP in the micropipette if simple diffusion of the cAMP is used to create the gradient. If the micromanipulator is equipped with a pump creating positive pressure to push cAMP out of the micropipette, much lower cAMP concentrations can be used.

17. The micropipette is extremely fragile and will break upon touching the coverslip. It is important not to break it because then the cAMP that will come out will cause a stimulation of the cells, and the resulted wider micropipette opening will create a different gradient. Therefore, for reproducibility, one should not use a broken micropipette. To avoid breaking it, keep the focus on the tip while finely lowering it, until the cells start being visible. You can then focus on the cells, and the micropipette tip should remain slightly out of focus.
18. If cells reach the tip and it is low enough, cells can then hit it, burst, and leave pieces of membranes in it and, thereby, block it. Consequently, the micropipette will not be usable anymore. If cells reach the pipette, it is best to stop the recording and bring the micropipette "home." The micropipette can then be reused for another recording in another area of the dish and/or for another dish.
19. We find that measuring the fluorescence in the cytosol, instead of measuring the fluorescence at the plasma membrane using kymographs, provides much more accurate measurements and reproducible results. The recruitment of the RBD-GFP to active Ras/Rap at the plasma membrane is then proportionally reflected by the loss of cytosolic fluorescence.
20. Since the basal intensity mostly reflects the expression level of the RBD-GFP, it is best to normalize the data and express as fold change over basal to compare between the conditions.

References

1. Weeks G, Spiegelman GB (2003) Roles played by Ras subfamily proteins in the cell and developmental biology of microorganisms. Cell Signal 15:901–909
2. Eichinger L, Pachebat JA, Glockner G, Rajandream MA, Sucgang R, Berriman M, Song J, Olsen R, Szafranski K, Xu Q, Tunggal B, Kummerfeld S, Madera M, Konfortov BA, Rivero F, Bankier AT, Lehmann R, Hamlin N, Davies R, Gaudet P, Fey P, Pilcher K, Chen G, Saunders D, Sodergren E, Davis P, Kerhornou A, Nie X, Hall N, Anjard C, Hemphill L, Bason N, Farbrother P, Desany B, Just E, Morio T, Rost R, Churcher C, Cooper J, Haydock S, van Driessche N, Cronin A, Goodhead I, Muzny D, Mourier T, Pain A, Lu M, Harper D, Lindsay R, Hauser H, James K, Quiles M, Madan Babu M, Saito T, Buchrieser C, Wardroper A, Felder M, Thangavelu M, Johnson D, Knights A, Loulseged H, Mungall K, Oliver K, Price C, Quail MA, Urushihara H, Hernandez J, Rabbinowitsch E, Steffen D, Sanders M, Ma J, Kohara Y, Sharp S, Simmonds M, Spiegler S, Tivey A, Sugano S, White B, Walker D, Woodward J, Winckler T, Tanaka Y, Shaulsky G, Schleicher M, Weinstock G, Rosenthal A, Cox EC, Chisholm RL, Gibbs R, Loomis WF, Platzer M, Kay RR, Williams J, Dear PH, Noegel AA, Barrell B, Kuspa A (2005) The genome of the social amoeba Dictyostelium discoideum. Nature 435:43–57
3. Kortholt A, van Haastert PJ (2008) Highlighting the role of Ras and Rap during Dictyostelium chemotaxis. Cell Signal 20:1415–1422
4. Park B, Kim H, Jeon TJ (2018) Loss of RapC causes defects in cytokinesis, cell migration, and multicellular development of Dictyostelium. Biochem Biophys Res Commun 499: 783–789

5. Kae H, Lim CJ, Spiegelman GB, Weeks G (2004) Chemoattractant-induced Ras activation during Dictyostelium aggregation. EMBO Rep 5:602–606
6. Kang R, Kae H, Ip H, Spiegelman GB, Weeks G (2002) Evidence for a role for the Dictyostelium Rap1 in cell viability and the response to osmotic stress. J Cell Sci 115:3675–3682
7. Charest PGG, Shen Z, Lakoduk A, Sasaki ATT, Briggs SPP, Firtel RAA (2010) A Ras signaling complex controls the RasC-TORC2 pathway and directed cell migration. Dev Cell 18:737–749
8. Fey P, Kowal AS, Gaudet P, Pilcher KE, Chisholm RL (2007) Protocols for growth and development of Dictyostelium discoideum. Nat Protoc 2:1307–1316
9. Kortholt A, Keizer-Gunnink I, Kataria R, Van Haastert PJ (2013) Ras activation and symmetry breaking during Dictyostelium chemotaxis. J Cell Sci 126:4502–4513
10. Kortholt A, Bolourani P, Rehmann H, Keizer-Gunnink I, Weeks G, Wittinghofer A, Van Haastert PJ (2010) A Rap/phosphatidylinositol 3-kinase pathway controls pseudopod formation [corrected]. Mol Biol Cell 21:936–945
11. Scavello M, Petlick ARARAR, Ramesh R, Thompson VFVF, Lotfi P, Charest PGPG (2017) Protein kinase A regulates the Ras, Rap1 and TORC2 pathways in response to the chemoattractant cAMP in Dictyostelium. J Cell Sci 130:1545–1558