[35] Imaging Spatiotemporal Dynamics of Rac Activation *in Vivo* with FLAIR

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Introduction

FLAIR (*FL*uorescent *A*ctivation *I*ndicator for *R*ho GTPases) is a biosensor system that maps the spatial and temporal dynamics of Rac activation in living cells. The approach is based on microinjection of a fluorescently labeled domain from p21-activated kinase into cells expressing green fluorescent protein (GFP)–Rac. The injected domain (called PBD, for p21-binding domain) binds only to Rac–GTP, and not to Rac–GDP.[1,2] Within living cells, PBD binds to the GTP–Rac wherever it has bound GTP, bringing the Alexa 546 dye (Molecular Probes, Eugene, OR) on the PBD near the GFP on the Rac to produce fluorescence resonance energy transfer (FRET). Thus the FRET signal marks subcellular locations where Rac is activated. This can be quantified to follow the changing levels and locations of Rac activation or to trace the kinetics of total Rac activation on an individual cell basis.

The labeling of PBD with Alexa and mammalian expression vectors for the expression of Rac–GFP are described in detail elsewhere.[3] This article describes a detailed protocol for the production of pure PBD, as this required considerable optimization. It then discusses how to generate cell images suitable for quantitative analysis of Rac activation and finally gives procedures and caveats for generating two types of data: images showing the spatial distribution of Rac activation within cells and curves showing the kinetics of Rac activation in single cells.

PBD Expression and Purification

PBD is expressed in the form of C-terminal hexahistidine (His_6) fusion from the prokaryotic expression vector pET23 (Novagen, Madison, WI). It was determined experimentally that the highest levels of expression are observed when a vector containing plain T7 promoter (not T7*lac*) is used

[1] G. Thompson, D. Owen, P. Chalk, and P. Lowe, *Biochemistry* **37,** 7885 (1998).
[2] V. Benard, B. P. Bohl, and G. M. Bokoch, *J. Biol. Chem.* **274,** 13198 (1999).
[3] V. S. Kraynov, C. E. Chamberlain, G. Bokoch, M. Schwartz, S. Slabaugh, and K. M. Hahn, submitted for publication.

0076-6879/00 $30.00

in combination with a BL21(DE3) strain [not the more stringent BL21(DE3)pLysS] of *Escherichia coli.* This system allows for "leaky" protein expression (Novagen). While the His_6 tag can be cleaved from the purified protein with thrombin, it is not necessary, as the tag does not have any significant effect on probe functionality.

Competent BL21(DE3) cells (Stratagene, La Jolla, CA) are transformed with pET23-PBD according to standard protocols[4] and plated on an LB plate containing carbenicillin. Cells do not degrade carbenicillin as quickly as ampicillin, so a higher percentage of cells retain the vector at the culture density appropriate for induction (Novagen). Five milliliters of LB media with 100 μg/ml carbenicillin is inoculated with a single colony of cells and grown in the shaker at 37° for 6–8 hr (until dense). Two milliliters of this is then used to inoculate 50 ml of LB*carb.* The rest of the culture is diluted 1:1 with glycerol and frozen for long-term storage at −80°. The 50-ml culture is incubated in the shaker overnight at 37°. The next morning, 1–2 liter of LB*carb* is inoculated with the overnight culture (15- to 20-ml culture/500 ml media) and grown in the shaker (37°) to OD_{600} of 0.8–0.9 (about 2–3 hr). The cultures are then chilled briefly on ice to 30°–32° and put back in the shaking incubator turned down to 30°–32°. The protein is expressed at a lowered temperature to increase the portion of the correctly folded, soluble PBD. Isopropylthiogalactoside (IPTG) is added to a final concentration of 0.4–0.5 m*M*, and the cultures are allowed to grow for another 4–5 hr at 30°–32° (shaker). The cells are collected by centrifugation (8000*g*, 4 min) and stored as a pellet at −20° until use. Approximately 2.5–3 g of cells is usually obtained from each liter of culture.

Purification of PBD-His_6 is performed essentially as described in the Talon affinity resin manual (Clontech, Palo Alto, CA). The cells (3–5 g) are thawed in 20–30 ml of the lysis buffer [30 m*M* Tris–HCl, pH 7.8, 250 m*M* NaCl, 10% (v/v) glycerol, 5 m*M* $MgCl_2$, 2 m*M* 2-mercaptoethanol (2-ME), 1 m*M* phenylmethylsulfonyl fluoride (PMSF)], homogenized with a spatula, and sonicated (four pulses, 10–15 sec each). T4 lysozyme and DNase are added in catalytic amounts (approximately 100 μg/ml lysozyme and 500 U DNase) to help the lysis, and the suspension is incubated on ice with periodic mixing for 30 min. The cells are then centrifuged at 12,500 rpm for 30 min, and the supernatant containing PBD is carefully transferred into a 50-ml Falcon tube.

The Talon resin (1.5–2 ml) (Clontech) is washed twice with 10 volumes of the lysis buffer in a 15-ml Falcon tube, centrifuging in the swinging bucket centrifuge at low speed in between to separate the resin. The cell

[4] J. Sambrook, E. F. Fritsch, and T. Maniatis, *in* "Molecular Cloning: A Laboratory Manual" (C. Nolan, ed.), p. 18. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

lysate is added to the 1.5 ml of washed Talon resin in a 50-ml Falcon tube and inverted or agitated gently (i.e., with an orbit shaker) for 20–30 min at room temperature.

The resin is then separated by centrifugation in a swinging bucket centrifuge. The supernatant containing the unbound fraction is removed and saved for SDS–PAGE analysis. The resin is then transferred into a new 15-ml Falcon tube and washed twice (10–15 min each, room temperature, orbit shaker) with 12 ml of the lysis buffer, without PMSF and 2-ME. The third wash is performed with lysis buffer + 10 m*M* imidazole (add 1 *M* stock in water, kept at −20°). After the final separation, the resin is resuspended in 2–3 ml of lysis buffer with 10 m*M* imidazole and pipetted into a column (0.5 cm in diameter). The resin is allowed to sediment by gravity flow until the fluid above the resin bed is almost gone, and then another 3–5 ml of lysis buffer with 10 m*M* imidazole is added to wash the column. Elution is performed using lysis buffer with 60 m*M* imidazole, and ca. 500-μl fractions are collected. PBD usually elutes in fractions 5–13 (total volume about 3–4 ml). An aliquot of each fraction is run on a 12% SDS–PAGE, and fractions containing pure PBD are combined and dialyzed against 1 liter of 25 m*M* NaP buffer (pH 7.3). A dialysis bag (SpectraPor 7) or dialysis cassette (Pierce, Rockford, IL) with a molecular weight cutoff value of 3,500 can be used.

After 2–3 hr of dialysis, the bag is wiped with a KimWipe and buried in Aquacide powder (Calbiochem) for 15–45 min at 4°, depending on the volume of the sample in the bag. This concentration process should be monitored carefully as complete drying may occur if the bag is left in the Aquacide for too long. The powder is scraped gently from the bag every 10–15 min to facilitate water absorption. When the sample reaches 0.5–1.5 ml in volume (3- to 10-fold concentration), the Aquacide is cleaned from the bag and the sample is removed carefully. The sample is centrifuged briefly (14,000 rpm, 2 min) to separate it from the precipitated material and transferred into a new dialysis bag or cassette. After the second dialysis step, the concentration of PBD is measured by taking a small aliquot (5–10 μl) and diluting into 50 m*M* Tris–HCl (pH 7.5–8.0) or other appropriate buffer. The extinction coefficient of PBD at 280 nm is 8250 (estimated from the primary sequence). On average, 1.5–2 mg of PBD is obtained per liter of cell culture.

Other methods of concentrating PBD were found to be less effective. For instance, centrifugal concentrators require prolonged centrifugations and result in nonspecific adsorption of the small PBD protein to the membrane. It is essential to perform dialysis after concentration with Aquacide. This prevents the ionic strength of the resultant protein preparation from becoming too high before labeling. Low ionic strength conditions are prefer-

able to avoid excessive precipitation of the protein during attachment of the hydrophobic dye. As mentioned earlier, PBD labeling is described elsewhere.[3]

Using FLAIR to Examine Rac Nucleotide State in Cells

Loading GFP–Rac and Alexa–PBD in Cells

Cells are first transfected with GFP–Rac through nuclear microinjection. We use the EGFP variant (Clontech, Palo Alto, CA), which produces significantly brighter cells than wild-type GFP.[5] For microinjection of DNA and of PBD–Alexa, glass pipettes with a 1.0-mm outer diameter and a 0.50-mm inner diameter (Sutter) are pulled using a micropipette puller (Sutter Model P-87, Novato, CA) to make microinjection needles with tips of approximately 0.5 μm diameter. Rac–GFP cDNA is injected into Swiss 3T3 fibroblasts at 200 ng/μl using a constant needle pressure of approximately 100 hPa. DNA can be centrifuged prior to injection (20,000*g* for 15 min) to prevent clogging the needle.

Cells expressing GFP–Rac are microinjected with Alexa–PBD using a microscope with optics and illumination capable of revealing the GFP fluorescence (detection sensitivity is typically improved by using higher NA objectives and brighter light sources, such as a 100-W Hg arc lamp). Thus only GFP-expressing cells need to be injected. To reduce background fluorescence during injection and the following experiment, cells are placed in 1 ml of prewarmed Dulbecco's phosphate buffer solution (DPBS) containing 1000 mg/liter D-glucose and 36 mg/liter sodium pyruvate and supplemented with 0.2% bovine serum albumin (BSA), 1% L-glutamine, and 1% (v/w) penicillin–streptomycin. During injection and the following experiment, cells are mounted in a Dvorak live cell chamber (Nicholson, Gaithersburg, MD) preheated to 37° and maintained at 37° by a heated stage (20/20 Technology, Wilmington, NC). The microscope can be equipped with a motorized stage and shutter controls (Ludl, Hawthorne, NY) to monitor multiple stage positions in one experiment.

Cells that are barely expressing or expressing too much GFP–Rac are ignored. The former produce FRET too weak for recording, and in the latter, overexpressed Rac affects the biology of the cell. We have shown that cells expressing less than 300 intensity units (IU) do not display Rac-induced ruffling and altered morphology. The precise value of this cutoff will depend on the sensitivity of the imaging system and should be determined by each laboratory for a relevant biological behavior. A 100 μM solution of Alexa–PBD is centrifuged at 20,000*g* for 1 hr prior to injection and is then

[5] R. Heim, R. Y. Tsien, *Curr. Biol.* **6,** 178 (1996).

injected into the cytoplasm of cells expressing GFP–Rac. Lowering the needle into the region just adjacent to the nucleus seems to produce the best combination of efficient injection and cell health. After the injection, cells are placed back into the 37° incubator for 5–10 min to recover. Alexa–PBD could potentially act as an inhibitor of Rac activity, so controls are carried out showing that, for our imaging system, up to 1000 IU of Alexa–PBD does not inhibit induction of ruffling.

Imaging Rac Activation

Imaging experiments were performed in our laboratory using a Photometrics KAF1400 cooled charge-coupled device (CCD) camera and Inovision (Raleigh, NC) ISEE software for image processing and microscope automation. Although filters are undergoing further optimization, the best success to date has been achieved with the following filters designed with sharp cutoffs specifically for this purpose by the Chroma corporation (Brattleboro, VT): GFP, HQ480/40, HQ535/50, Q505LP; FRET, D480/30, HQ610/75, 505DCLP; and Alexa, HQ545/30, HQ610/75, Q565LP.

The exact camera settings depend on the type of experiment being performed. When the total Rac activity within the cell is being determined, images are not generated, so spatial resolution can be sacrificed for increased sensitivity. Images are taken using 3×3 binning with exposure times of 0.1, 0.1, and 0.5 sec for GFP, Alexa, and FRET respectively. When images are required, i.e., to examine the changing spatial distribution of Rac activation, 1×1 binning is used with exposure times of 1, 1, and up to 5 sec for GFP, Alexa, and FRET respectively. These settings depend on the sensitivity of the imaging system used and the desired trade-off between sensitivity and spatial or temporal resolution. Settings should always be chosen not to exceed the dynamic range of the camera.[6] Another important consideration during imaging experiments are motion artifacts. For fast-moving phenomena, features of the cell may move appreciably during the time between acquisition of the FRET and GFP images. This results in artifacts when the image is corrected for bleed through, as described in more detail later. This can only be prevented by reducing the time between exposures or by more expensive solutions, such as using two cameras simultaneously.

When total Rac activation is being determined, a picture of GFP–Rac and FRET is taken at each time point. Only one image of Alexa–PBD, usually at the initial time point, will also be required (for bleed-through corrections as described later). In contrast, when generating images (i.e., to determine the distribution of rac activation), an Alexa–PBD image must

[6] K. Berland and K. Jacobson, *in* "Video Microscopy" (G. Sluder and D. Wolf, eds.), p. 33. Academic Press, San Diego, 1998.

be taken at each time point. The reasons for this are discussed in the following section. If the cells are to be treated with some type of stimulus, it is helpful to take a series of images prior to stimulation as controls for noise, bleaching, and other artifacts.

Image Processing

Image analysis as described here is performed to follow the kinetics of total Rac activity within individual cells or to generate images that show the subcellular location of Rac activation. It is easy to generate artefactual data during this process, so this section emphasizes the proper application of corrections essential for quantitative imaging. We use common image processing operations whose precise implementation will depend on the software package used.[6] The correction factors must be applied rigorously when using the FLAIR system, as FRET signals will be low relative to other sources of fluorescence in the sample. The FRET signal must purposefully be kept low, as minimum quantities of fluorescent molecules should be used to prevent perturbation of cell behavior. Although the operations described are similar to those used for other fluorescent probes, there are important differences unique to application of FLAIR.

The following protocols assume use of a cooled CCD camera, which typically shows low levels of noise, linear response to light intensity, and little variation in response from pixel to pixel. It is valuable to use cameras and software with the greatest possible bit depth (allotment of computer memory to each pixel to maximize the number of possible intensity gradations). This is especially important for ratio operations, which are typically performed using 12-bit images or greater. Operations are described in the order in which they should be performed.

Registration

For corrections applied in later steps, it is important that each of the images taken using different excitation and emission wavelengths be registered so that the cells lie atop one another, with cell edges and internal features exactly coinciding. Different image processing software will accomplish this in different ways, but it usually involves manual translocation of one image so that it lines up with a second, fixed image. This is best accomplished by zooming in on cells and adjusting brightness and contrast to clearly see cell edges and internal features. Because the GFP–Rac signal is strongest in our experiments, it is used as the reference image. Errors in registration often become apparent as "shadow effects" when the bleed-

through corrections described later are performed (see error illustrated in Fig. 1).

Background Subtraction

There are two methods commonly used for background subtraction. If the only intention is to follow the changing level of the FRET signal over time, and if the background (in the absence of cells) remains uniform across the field of view throughout the experiment, then it is sufficient to determine the background intensity in several regions of the image outside the cell. The average value of these intensities is then subtracted from each pixel in the image. This method can also be sufficient for following qualitative changes in the subcellular location of activation, but it must be used with caution. Subtle variations in background intensity across the cell could be large relative to the changes observed in FRET, producing significant artifacts. When quantifying the kinetics of total cell Rac activity over time

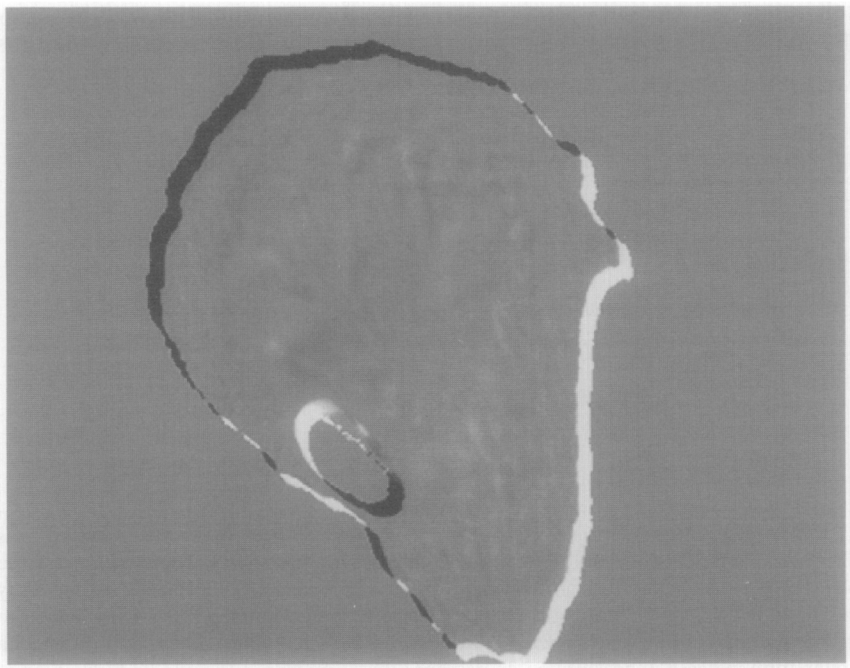

FIG. 1. Registration artifact. Before bleed-through correction, in which one image is subtracted from another, it is important to superimpose the two images precisely. Small differences in the microscope optics shift the position of one image relative to the other. If registration is performed incorrectly, a characteristic edge effect is seen, in which bleed-through correction results in artifactual bright and dark rims on exactly opposite sides of objects and cell edges.

(see following sections), it is better to take an image of a region of the coverslip containing no cells or fluorescent debris under the same conditions used for taking the real image. This background image is then subtracted from the real image prior to further analysis. A separate background image must be taken for each type of image (GFP, FRET, or Alexa) and at each time point when successive images are obtained. Artifacts that can be generated using the simpler background subtraction during quantitative analysis are illustrated in Fig. 2.

Masking

It is valuable to mask out regions surrounding the cells prior to further analysis. The edges of the cell are outlined in most software either manually or by eliminating all sections of the images below a certain intensity value (interactive threshholding). Regions outside the cell are thus identified and eliminated from further calculations. (The precise approach will depend on software. The mask is usually a binary image with all values within the cell equal to 1 and all outside equal to 0. The real image is multiplied by the mask.) The mask is best generated using the GFP image, which has

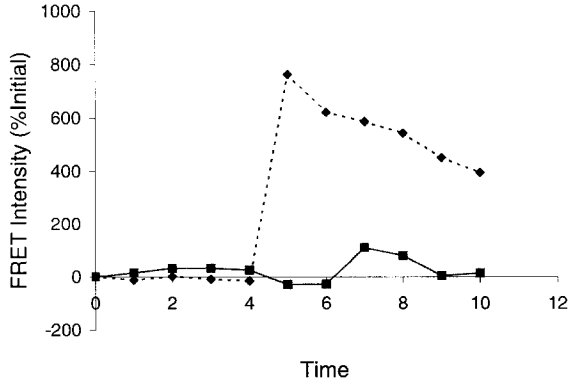

FIG. 2. Background subtraction artifacts. If the efficiency of fluorescence detection is uneven across the field of view (i.e., uneven illumination, poorly corrected objectives, refractile objects), subtraction of a background image is superior to simply subtracting the intensity measured in an "empty" area of the image. In the example shown here, serum is added to a coverslip that has uneven illumination and poorly corrected optics. The serum produces a nonuniform increase in background fluorescence that is greater in the middle of the field of view. If the background is simply taken as intensity near the edge of the field, an artifactual increase in intensity will be observed after background subtraction (dashed line). When one obtains an actual image from a region of the coverslip that has no cells and subtracts this image, no artifactual intensity increase is seen (solid line). These effects are exaggerated when the real signal is not very large relative to the background.

the strongest signal and therefore the most clearly defined edges. When determining the total intensity within the cell, analysis should be carried out on the same pixels within the FRET, GFP, and Alexa images. Therefore, the same mask is applied to each image after registration, assuring that exactly the same pixels are analyzed.

Bleed-Through Correction

During FRET it is necessary to excite the donor fluorophore while monitoring emission from the acceptor fluorophore. It is extremely difficult to design FRET filters that see only FRET emission and block all GFP emission or block all light from Alexa excited directly rather than by FRET. To correct for "bleed-through" of such light into the FRET image, the fluorescence filters must be characterized by taking images of cells containing GFP–Rac or Alexa–PBD alone. For example, in bleed-through correction for GFP, cells are imaged using both the GFP and the FRET filter set. When observing GFP fluorescence through FRET filters, a fixed percentage of GFP emission will be seen. The total fluorescence intensity is determined for both GFP and FRET images from cells containing only GFP–Rac. A GFP bleed-through factor is computed for each cell by dividing the intensity through the FRET filters by that through the GFP filters (the "bleed-through factor" for GFP: FRET intensity/GFP intensity). This value is plotted against cell intensity for numerous cells, and a line is fit to these data to produce an accurate value of the bleed-through factor. It is important to use background-subtracted images. The process is repeated for Alexa–PBD. When the actual experiment is performed, Alexa–PBD, GFP–Rac, and FRET images are obtained. After background subtraction of all three images, the Alexa–PBD and GFP–Rac images are multiplied by the appropriate bleed-through factor and subtracted from the FRET image. This is an extremely important step, which must be applied carefully to prevent artifacts that appear to be regions of high FRET, especially as the magnitude of the FRET signal approaches that of the bleed-through. It is important not to use GFP–Rac or Alexa–PBD images that exceed the dynamic range of the camera ("overexposure") as they will not fully eliminate bleed-through. Motion artifacts (see earlier discussion) can also produce errors derived from bleed-through corrections, as illustrated in Fig. 3.

Production of Total Activation Curves (Bleaching Correction)

To determine how overall Rac activity within a cell changes over time, the total fluorescence intensity within a GFP–Rac and FRET image is determined at each time point. The intensity of the FRET image is divided

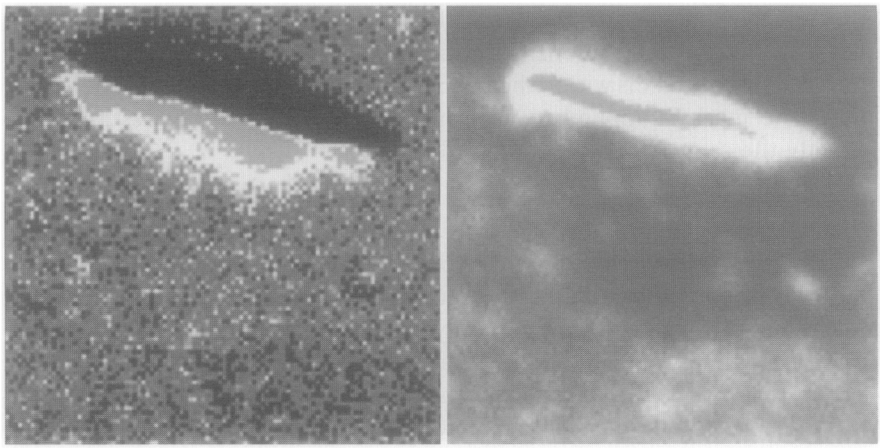

FIG. 3. Motion artifact. (Left) The FRET channel and (right) the GFP channel after corrections for bleed-through have been applied. In bleed-through correction, the GFP image is reduced in intensity by a precise percentage and is then subtracted from the FRET image to eliminate GFP emission being transmitted by the FRET microscope filters (see text). Because the object being imaged moved between acquisition of the GFP and FRET images, bleed-through subtraction produced an artifactual dark area in the FRET image and an artifactual localized increase in FRET.

by that of the GFP–Rac image. This ratio better reflects total Rac activity than FRET intensity alone. Division by GFP–Rac normalizes out errors due to bleaching of GFP, effects of uneven illumination, and other factors affecting both GFP and FRET signals. Because all FRET occurs through irradiation of GFP, bleaching of GFP will decrease both GFP and FRET emissions to the same extent. Therefore, the FRET/GFP ratio will be a measure of Rac activity that is not affected by bleaching. It is critical that each image be properly background subtracted and corrected for bleed-through. Bleed-through corrections are somewhat simplified when generating these curves, as they need not be carried out on actual images, but simply on the total intensity values derived from the images. The total intensity of the GFP–Rac image is multiplied by the GFP bleed-through factor, and the resulting value is subtracted from the FRET intensity. An analogous operation is performed for Alexa–PBD bleed-through. One need only obtain a single Alexa–PBD image (usually at the beginning of the time series) and use this for bleed-through correction of all images in the time series. If Alexa–PBD is not irradiated during the experiment, its bleaching will be negligible, and this single image will reflect the actual Alexa–PBD level throughout the entire time series.

Any ratio calculations are best performed using floating point opera-

tions. Large errors can be generated by software that truncates noninteger values into integers to display data as images. Such problems can be overcome by multiplying the images by a large scalar value prior to division. This value should be as high as possible without causing any pixel to exceed the bit depth of the image file. For example, a 12-bit image file can only hold values up to 4095 (2^{12}), so the constant selected must not cause the highest intensity value in the image to exceed 4095. When operations are performed on whole cell values rather than on images, as with production of the Rac activation curves described here, it is convenient to determine total cell intensities and then perform any division operations in a floating point spreadsheet program, such as Microsoft Excel.

Examining Changes in Localization of Rac Activation

The preceding sections described how to generate images showing the distribution of FRET within cells. A sequence of such images can be compared to show how localizations vary with time. While simple examination can suffice to show distribution changes, the overall intensity of FRET, and hence the perceived activation level, could become successively lower over time due to bleaching of the GFP. We have found that bleaching is not a serious issue for at least 20 images under the exposure conditions described here. The total GFP intensity at the beginning and end of the experiment should be examined to gauge bleaching effects. One can correct for bleaching by dividing each pixel in the image by the total intensity of GFP determined at the same time point.

Concluding Remarks

Many aspects of cytoskeletal control and signaling crosstalk depend on the localization of Rho family GTPase activation and may depend as well on the level and duration of activation. Signaling control by the precise dynamics of GTPase activation has been suggested by indirect experiments, but has been very difficult to quantify or study using previous methods. The FLAIR system can reveal Rac activation dynamics *in vivo*. We are currently working toward making FLAIR more readily accessible, either by using only genetically encoded fluorophores, such as GFP mutants, or by using peptide import sequences to simplify loading of the domain. The greatest benefits will be derived from accurate quantitation of Rac–GTP levels. The FLAIR system as described here can accurately report the changing activation levels within one cell, but the fluorescence response to the same level of Rac–GTP will vary from cell to cell. We are trying now to standardize the approach, eliminate this variability, and develop a system

permitting quantitative comparisons of different individual cells or of different cell populations assayed simply in a fluorometer cuvette. It is hoped that FLAIR is an example of a generally applicable technology that can benefit researchers studying other protein families.

Acknowledgments

We are grateful to the Chroma Corporation for their outstanding help in designing filters optimized for FLAIR, to the Inovision corporation for their expert technical assistance and help in software design, and to Ella Blanc for expert secretarial assistance. We thank the National Institutes of Health (R01 GM-57464) and the Arthritis Foundation for financial support.