Fluorescence Localization After Photobleaching (FLAP)

Fluorescence localization after photobleaching (FLAP) is a new method for localized photolabeling and subsequent tracking of specific molecules within living cells. The molecular species to be located carries two different fluorophores that can be imaged independently but simultaneously by fluorescence microscopy. For the method to work, these two fluorophores should be accurately colocalized throughout the cell so that their images are closely matched. One of the fluorophores (the target fluorophore) is then rapidly photobleached at a chosen location. The unbleached (reference) fluorophore remains colocalized with the target fluorophore; thus the subsequent fate of the photobleached molecules can be revealed by processing simultaneously acquired digital images of the two fluorophores. For example, an absolute FLAP image, which shows the location of the photobleached molecules, is calculated simply by subtracting the target intensity from the reference intensity at each pixel. This image is effectively the same as images obtained directly by photoactivation methods, so absolute FLAP can be considered to be a method of virtual photoactivation. In addition, however, a relative FLAP image can be calculated to show the photobleached fraction of molecules within each pixel. This useful information is not available with other methods.

The Basic Protocol in this unit demonstrates the simplicity and effectiveness of the FLAP method in revealing both fast and slow molecular dynamics in living cells. As an example, cDNA fusion constructs of β-actin with yellow and cyan fluorescent proteins (YFP and CFP) are microinjected into the nuclei of transformed rat fibroblasts. Using a Zeiss LSM 510 laser-scanning microscope, the authors show that it is possible to follow simultaneously the fast relocation dynamics of monomeric (globular) G-actin and the much slower dynamics of polymeric (filamentous) F-actin in expressing cells.

In order to give a step-by-step protocol for the FLAP method, it is necessary to focus on a particular configuration of fluorophores and microscopy methods. CFP-actin and YFP-actin fusion proteins have been chosen as the fluorescent molecules, since YFP can be efficiently and rapidly photobleached. Moreover, the predominance of native actin in the cell means that noise-free images can be obtained at low relative expression levels of the tagged molecules. Using wide-field fluorescence microscopy, it is possible to separate the emission spectra of these two fluorophores and thus obtain simultaneous images. For this, both fluorophores are excited simultaneously, and a commercially available arrangement of fixed dichroic mirrors and filters (Cairn Research Ltd.) enables the two images to be projected side-by-side onto a single CCD chip. However, in view of the large overlap of emission spectra of CFP and YFP, laser scanning microscopy, which permits alternately exciting the two fluorophores line-by-line and yields images that are effectively simultaneous, has been chosen for the Basic Protocol. Some laser scanning microscopes, such as the Zeiss LSM 510 used here, have the further advantages of versatile choice of size, shape and location of the bleach region, and rapid resumption of image scanning after bleaching. Time-lapse recording using no intervals between scanned frames will reveal rapid diffusion dynamics, whereas intervals of several seconds or longer may be required to reveal the movements of polymerized or bound molecules without excessive fading of the fluors.

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FLAP OF ACTIN IN LIVING CELLS

This protocol describes the FLAP method using transformed rat fibroblasts of the lines K2 and T15 (Pavel Vesely, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Flemingovo nam. 2, 166 37 Prague 6 Dejvice, Czech Republic; e-mail, *pvy@zeus.img.cas.cz*). Many other cell types that the authors have tried work equally well. A notable exception is the Swiss 3T3 line, which shows significant autofluorescence on the YFP channel. Extensive details on preparation of constructs, cell culture, and microinjection methods will not be given, as these are standard procedures. Also a basic familiarity with the software and operation of the Zeiss LSM 510 upright microscope will be assumed. Once the microscope and software have been prepared (Support Protocol 1), there are two main sequential stages to the Basic Protocol: (1) preparation of cells and setting up the microscope (steps 1 to 4) and (2) microscopy (steps 5 to 17). Post-processing and analysis of images (Support Protocol 2) can be performed later.

Materials

Rat fibroblast cell line K2 or T15
Hanks' Minimal Essential Medium (MEM; Cancer Research UK; *daniel.zicha@cancer.org.uk*) containing 10% bovine serum and no antibiotics
cDNA constructs of eCFP-β-actin and eYFP-β-actin (see recipe)
Experimental reagents of interest (e.g., myosin light chain kinase inhibitor, ML-7)
Hot wax mixture (see recipe)
Non-toxic immersion oil optimized for 37°C, refractive index 1.515 (Cargille Labs)
18 × 18–mm glass coverslips
35-mm plastic petri dishes (Costar)
Microinjection system (also see *UNITS 4.10 & 17.1*) including:
  5171 micromanipulator (Eppendorf)
  5246 transjector (Eppendorf)
Zeiss Axiovert 35 microscope
Microneedles (GC120TF-10, Harvard Apparatus)
P97 Flaming/Brown micropipette puller (Sutter)
Optical chambers (see recipe)
Zeiss upright LSM 510 microscope (see Support Protocol 1 for full configuration) contained within a 37°C environmental control incubator (e.g., Microscope Temperature Control System, Life Imaging Services) *or* a similar apparatus assembled in house (Fig. 21.2.1)
Software:
  Zeiss LSM 510 operating software for image acquisition
  Zeiss LSM Reader for image review (free download; see Internet Resources)
Additional reagents and equipment for cell culture (*UNIT 1.1*), microinjection (see *UNITS 4.10 & 17.1*), and use of LSM 510 operating software (see Support Protocol 1)

NOTE: All solutions and equipment coming into contact with living cells must be sterile and aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) require altered levels of CO_2 to maintain pH 7.4.

Prepare cells and set up microscope

1. Seed cells at the required density onto the 18 × 18–mm coverslips in 35-mm plastic petri dishes and flood with Hanks' Minimal Essential Medium/10% bovine serum (see *UNIT 1.1* for basic cell culture techniques). Incubate 72 hr.

Figure 21.2.1 Zeiss upright LSM 510 microscope contained within a 37°C environmental control incubator. In the authors' laboratory, the enviromental control chamber is a specially built Plexiglass box with access hatches (Cancer Research UK workshop). The essential components for the heater and control system are a centrifugal fan (part no. 40BTFL from Air Flow Developments), and a temperature controller (208-2739), thermocouple type T (219-4680), box (584-615), mini-3-pole plug (449-269), mini-3-pole socket (449-275), fuse holder (418-603), optical relay (394-535), type T panel socket (219-4860), type T line plug (219-4876), enclosed heater (224-565), and thermocouple connector (219-4876), all from RS Components. A similar commercially available system (Microscope Temperature Control System) may be obtained from Life Imaging Services (see Internet Resources).

MEM has been optimized by the authors at Cancer Research UK for the cell lines used here. However, it might be possible to obtain satisfactory results with the MEM formulation supplied by Sigma.

In the authors' laboratory, experiments usually require that the cell density still be well below confluence (~50%) after this incubation.

2. Microinject the two cDNA constructs at concentrations of 50 ng/μl (YFP-actin) and 75 ng/μl (CFP-actin) into the nuclei of 20 to 30 cells located near the center of each coverslip. Return to incubator for 2 to 3 hr.

   Expression and folding times may vary widely with other fluorescent protein constructs.

3. During the incubation, switch environmental heating and argon laser on the LSM 510 to on and open a database with previous software settings (see Support Protocol 1).

4. Assemble a coverslip culture (from step 2) onto an optical chamber filled with medium and any experimental reagents. Seal chamber with hot wax mixture, wash outside of chamber with clean distilled water, and blow dry before placing on the stage of the LSM 510.

Perform microscopy

5. Restore settings for tile scanning by selecting the previous tile scan from the saved database and reusing the settings (Support Protocol 1). Check that scan rate is now at fastest setting and laser power settings are correct.

Tile scanning provides a montage of adjacent fields acquired sequentially in order to overview a large area for identification of expressing cells.

6. Use the stage control joystick to locate the center of the chamber on the microscope axis, switch to direct viewing using a low-power phase objective (5× to 25×), change phase setting of condenser as necessary, and focus on cells manually.

7. Start Tile scan in Stage controls.

   The authors generally use a 4 × 4 tile array with 256 × 256 pixel images.

8. Mark the expressing cells. Create an image database and use Save As to store the tile scan.

9. Restore software settings for FLAP imaging by selecting a FLAP sequence from the saved database and reusing the settings (Support Protocol 1). Use the stage-control joystick to move the chamber away from objective, apply immersion oil, and recentralize chamber by moving to the first marked cell. Switch to high-aperture oil-immersion objective (63× Plan apochromat Ph3) and change phase setting of condenser as necessary.

   CAUTION: *Do not attempt to switch objectives automatically unless it has been ascertained that they are accurately parfocal (Support Protocol 1) and that the lower surface of the upper coverslip is in focus. The high-power objective may have a very short working distance and any error could cause a disastrous crash between the objective and the specimen.*

10. Start a fast scan while displaying split channels and manually focus on a cell.

    The interference reflection microscopy (IRM) image will brighten suddenly when one is focused on the lower surface of the upper coverslip. A small distance below this surface (turn top of fine focus knob away from you) will usually be the optimal setting with maximal brightness of the fluorescence images.

11. If necessary, centralize the cell in image field using the X/Y buttons and the nudge wheel on the joystick control. Cease fast scanning as soon as possible.

    Note that the IRM image will often be out of focus at the optimal setting for fluorescence imaging; a compromise is necessary if good IRM images are required.

12. Select orientation and zoom factor (the authors usually keep a standard zoom of 2) and do a single scan.

    When it is critical to record fast molecular dynamics immediately after bleaching, note that the top of the reoriented image will be scanned first.

13. Click the use ROI button to set up region of interest (ROI) if required in order to reduce scan times.

    It is sometimes advantageous to image the whole cell for accurate estimates of fluorophore fade during imaging (Support Protocols 2). Note that the top one or two scan lines will sometimes show a defective match between channels and should be avoided (see Troubleshooting).

14. Do a single scan while displaying split channels. Switch to the custom Hall palette (Support Protocol 1). Using a minimal number of further scans, adjust laser gains and offsets so that the two fluorescence channels are accurately matched with no saturation and with a low but finite background intensity. Adjust settings for the other two channels if necessary. Return to the no palette display.

    If matched, the fifth combined image should show the fluorescent regions as pure white (Fig. 21.2.2). The phase-contrast and IRM channels should show good contrast.

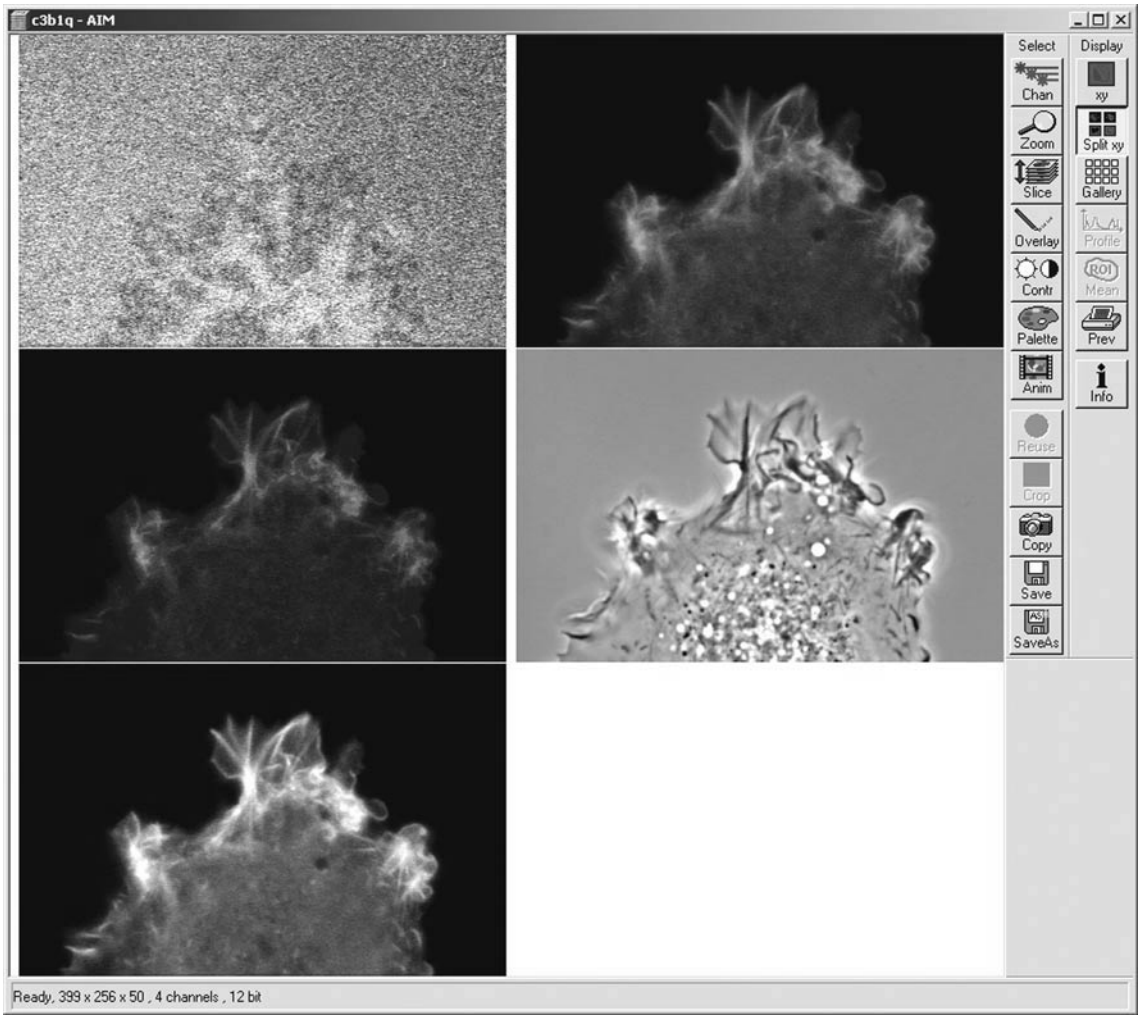

Figure 21.2.2 Window from Zeiss LSM software showing four channels and fifth combined channel after setting gains and offsets and laser powers. Courtesy of Carl Zeiss, Germany; reprinted with permission of Zeiss UK. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of the figure go to http://www.currentprotocols.com/colorfigures.*

15. Select the bleach panel, choose a bleach region, and change the bleach parameters if necessary.

    The authors use 50 bleach scans at the full-power laser setting. Note that bleach times depend on the height of the bleach region (regardless of its shape) but not on its width, so a horizontal narrow strip will bleach a large area rapidly.

16. Select the time series panel and change the time-lapse parameters if necessary. Do one single scan to check that everything is still OK (i.e., that the current setup still provides a satisfactory image of the selected cell). Choose the number of scans to average and start the recording.

    The authors normally average four scans per frame and record two frames before bleaching and 50 afterwards with no time-lapse interval. Recording of the FLAP signal is best monitored in a large window showing the combined image. After bleaching, the bleach-labeled molecules will then show as a bluish color against a white background (Fig. 21.2.3). Avoid transmitting any vibrations to the microscope during recording.

17. Save the recorded sequence if satisfactory, export the bleach region image for later reference (showing the bleach region and with the zoom aspect set to 1:1) as a

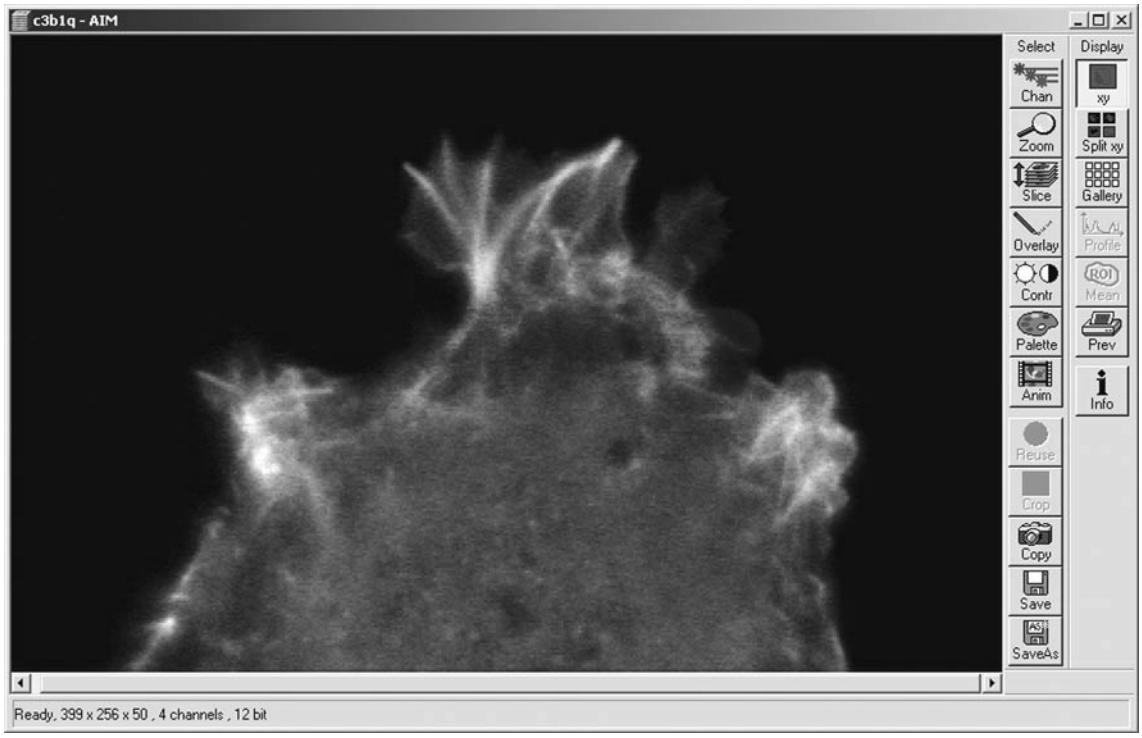

Figure 21.2.3 Window from Zeiss LSM software showing only the combined channel after bleaching during data recording. Courtesy of Carl Zeiss, Germany; reprinted with permission of Zeiss U K. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of the figure go to http://www.currentprotocols.com/colorfigures.*

single-image TIFF file, reset the scan averaging to 1, move to the next marked cell using the stage-control panel, and repeat steps 10 through 16 for all marked cells. Return to step 5 to start a new culture chamber.

SUPPORT PROTOCOL 1

SETTING UP THE LSM 510 AND ITS SOFTWARE

It is important that the microscope be configured optimally—e.g., pinhole alignment should be set correctly. Optimization of settings for tile scans and FLAP imaging will require a trial run through the Basic Protocol.

Materials

Zeiss upright LSM 510 microscope and software (see Basic Protocol 1)
Small beads (e.g., TetraSpeck microspheres, 0.2 μm; Molecular Probes)

1. Make sure that the objectives to be used are accurately parfocal using small beads and Parfocal Settings from the `CLM32.EXE` program.
2. Define the pseudocolor lookup table (LUT) for the custom Hall palette (Fig. 21.2.4) to be used for gain and offset adjustments and for viewing fully processed FLAP images.

   Other preferred LUTs may be used, but it is critical that minimum and maximum gray levels be easily distinguishable from the rest.

3. For tile scans, use three channels (CFP fluorescence, YFP fluorescence, and phase contrast), a low number of pixels (256 × 256), and fast scanning.

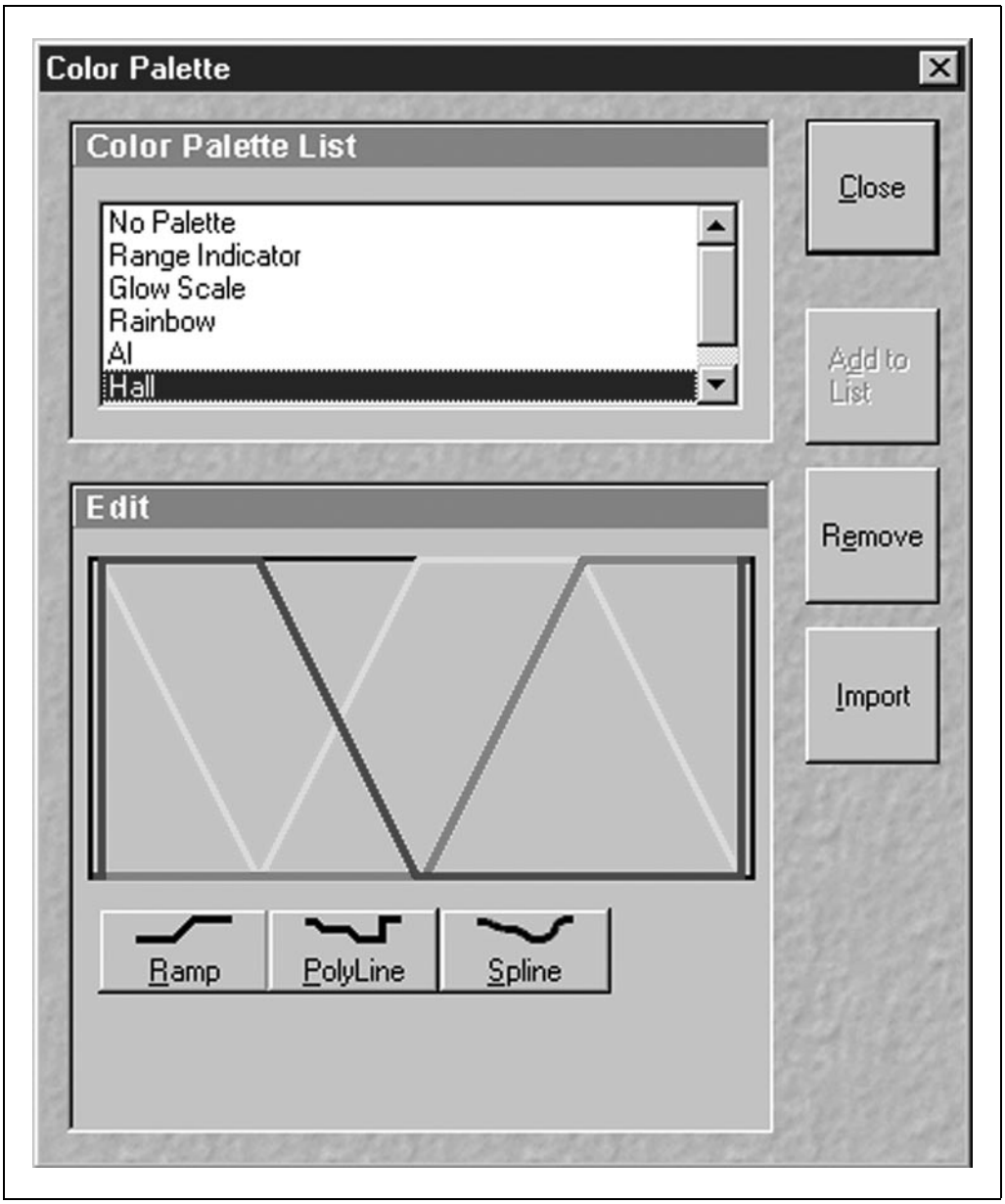

Figure 21.2.4 Window from Zeiss LSM software showing custom Hall pseudocolor palette. Note that the lowest intensity level is coded as black and the highest as white. Courtesy of Carl Zeiss, Germany; reprinted with permission of Zeiss UK.

4. For FLAP imaging, set the microscope to use 4 channels: CFP fluorescence, YFP fluorescence, phase contrast, and IRM (Fig. 21.2.5) with 512 × 512 pixels.

   Tile scan is used to identify expressing cells for FLAP imaging.

5. Set (typically) laser line 514 nm power to 1% and 548 nm power to 21%; pinhole diameters Ch1 to 408 μm; Ch2 to 409 μm; and Ch3 to 414 μm.

   These pinhole sizes give optical sections of 3 μm in all channels.

6. Set dichroic mirror HT to HTF 458/514; set dichoic mirror NT1 to NFT 635 VIS; set dichroic mirror NT2 to NFT 545; set dichroic mirror NT3 to Plate; set filter Ch1 to None for IRM; set filter Ch2 to BP 475-525 for CFP fluorescence; set filter Ch3 to LP 530 for YFP fluorescence; and set filter ChD for phase-contrast microscopy (Fig. 21.2.5).

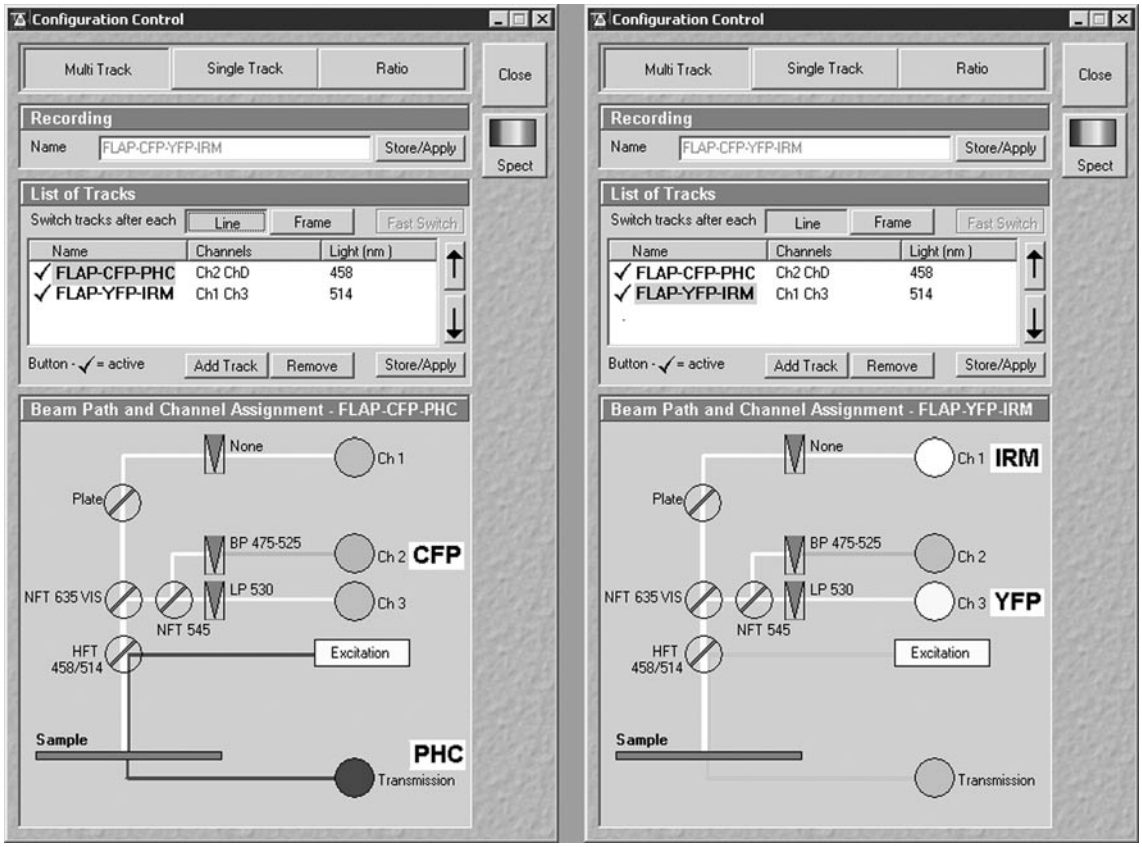

Figure 21.2.5 Window from Zeiss LSM software showing both tracks on the configuration panel. Courtesy˜of Carl Zeiss, Germany; reprinted with permission of Zeiss UK.

7. Set pixel size to 0.29 μm × 0.29 μm and pixel dwell time to 1.6 μsec.
8. Use laser line 514 nm under the bleach setup at maximum power measured as 1.32 mW.
9. Set palette of the CFP fluorescence channel to RGB = (0, 128, 255) and YFP fluorescence channel to RGB = (255, 128, 0) so that the cyan and yellow channels are easily identified and equal intensities combine to a white image when the phase contrast and IRM are switched off from the overlay panel.
10. Save settings by saving the trial run as a database for future Reuse. Do not check Re-use Objective in the Save Settings menu, to avoid unexpected movements of the objective revolver.

Further runs will be required to refine gain and laser power settings for fade matching of channels (Support Protocol 2).

SUPPORT PROTOCOL 2

IMAGE PROCESSING AND ANALYSIS

The authors perform the image processing in Mathematica (Wolfram Research). This has the advantage of immense flexibility over dedicated image-processing software and can be programmed to read the information in the `*.lsm` Zeiss file headers. A commercial image acquisition and processing program from Kinetic Imaging (*http://www.kineticimaging.com*) can also read these file headers as, of course, can the LSM Reader software distributed as a free download by Zeiss. The LSM Reader will also export the individual images of the four channels as 12-bit TIFF format files. The required

image processing is fairly basic, and it will be assumed that a package is available that can read and operate on the raw 12-bit TIFF files exported from LSM Reader.

Materials

Mathematica v 4.2 or 5 (Wolfram Research) or a dedicated image-processing package capable of processing 12-bit TIFF images

1. Subtract a constant from the intensity values of all the CFP and YFP images (it may be different for each image) so that the background regions outside the cell(s) have a mean intensity of zero. Set all negative intensity values to zero.

   The intensity values should now be proportional to the number of fluorescent molecules at each location if care was taken to avoid saturation in step 14 of the Basic Protocol.

2. Multiply the intensity values of the last pre-bleach YFP image by a factor so that the total image intensity matches that of the last pre-bleach CFP image.

   The two images should now look identical.

3. Multiply all the remaining YFP images by the factor obtained in step 2.

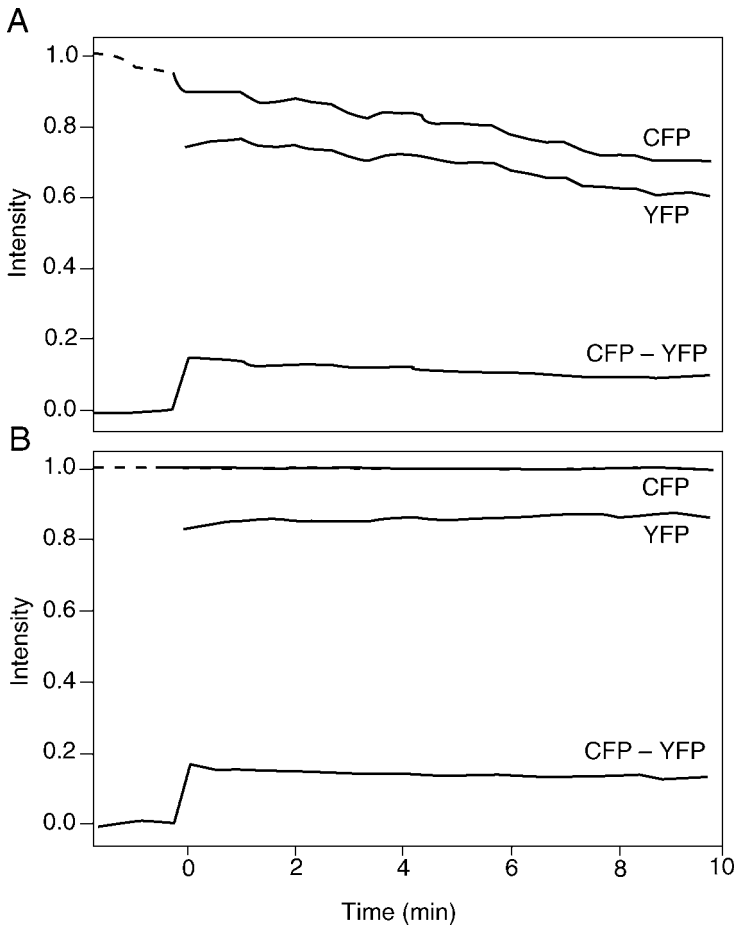

Figure 21.2.6 (**A**) The summed intensity values for a whole cell during a 10-min time series after step 3 in Support Protocol 2. These values have been normalized so that the total intensity of the first CFP image is 1. (**B**) The same intensity values after fade compensation as in step 4 of Support Protocol 2. This consists of dividing each CFP image by a factor so that the total intensity is 1 and then dividing the corresponding YFP image by the same factor. Note that fluctuations due to cyclical focus drift have also been compensated.

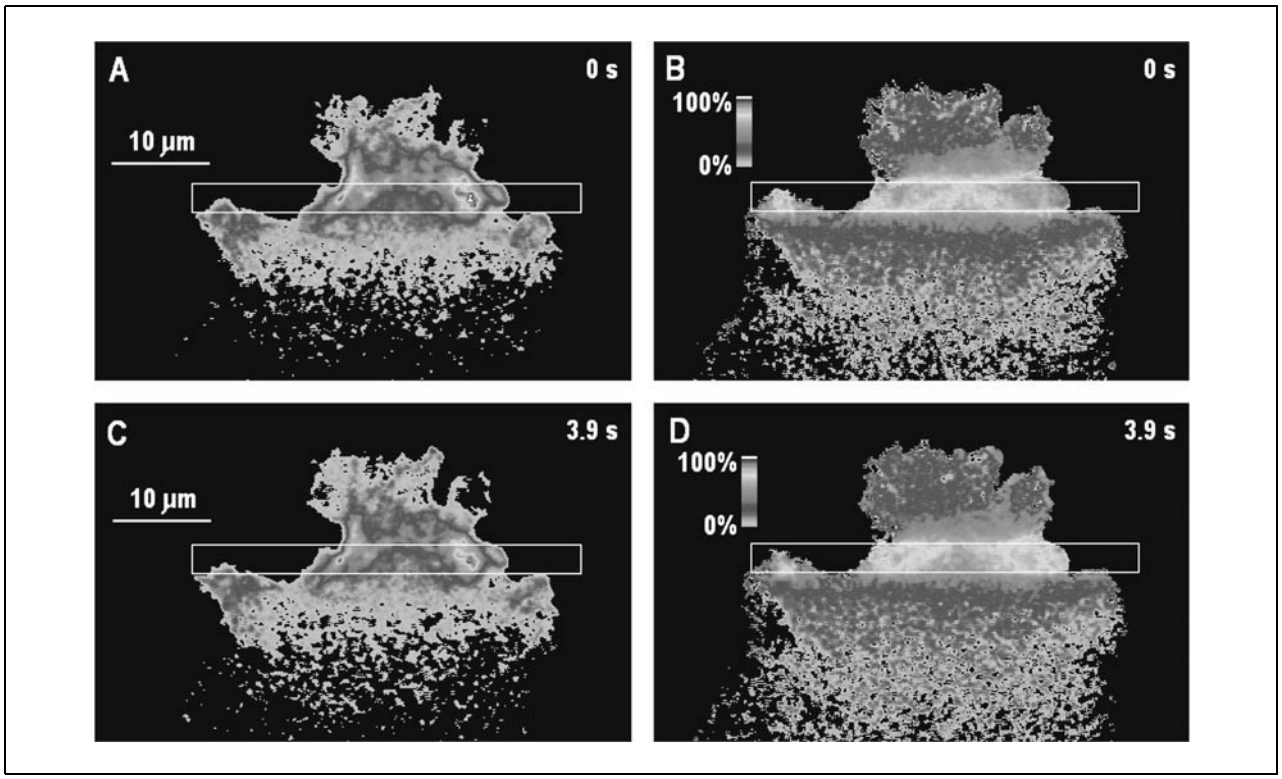

Figure 21.2.7 Absolute FLAP (**A, C**) and relative FLAP (**B, D**) images of the cell featured in Figures 21.2.2 and 21.2.3 recorded immediately after bleaching (**A, B**) and 3.9 sec later (**C, D**). Bleach box is shown as white rectangle. Hall palette. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of the figure go to http://www.currentprotocols.com/colorfigures.*

If the YFP and CFP channels do not fade at the same rate (see Support Protocol 1), it will be necessary to change this factor for each YFP image. This is best calculated if a whole cell is contained within the image. Plots of the total image intensity of each channel against time will then reveal the fade rates and any compensation that is necessary (Fig. 21.2.6A).

4. If planning to calculate absolute FLAP images, multiply all images after the first pair by a factor to compensate for fade.

   The fade rate is best calculated on whole cells as in step 3. After compensation, plots of the total image intensity of each channel against time should be horizontal lines with a step fall at bleaching in the YFP channel (Fig. 21.2.6B).

5. For each pair of simultaneous images, subtract the YFP image from the CFP image. Set all negative intensity values to zero.

   If fade compensation has been performed as in step 4, the resulting images are now absolute FLAP images.

6. Divide each image obtained in step 5 by the corresponding CFP image to obtain the relative FLAP images.

   Note that this operation has an infinite or undefined result for pixels in which the CFP intensity is zero and an unreliable result where intensity values are low. A threshold level of CFP intensity should be chosen (by trial and error) below which the result of this operation is set to zero.

7. View the final FLAP images, if desired, using a pseudocolor lookup table or palette.

   The authors often use the Hall palette (Support Protocols 1). The relative FLAP images (Fig. 21.2.7B) have intensity values in the range 0 to 1, and a scale bar showing the corresponding pseudocolors can be calibrated from 0% to 100%, showing the fraction

of bleached YFP molecules in each pixel. Absolute FLAP images (Fig. 21.2.7A) could in theory be calibrated for numbers of molecules, but this would require knowing numbers of expressed and native molecules for the whole cell. If the images are noisy and the noise levels in the raw images cannot be improved further (see Troubleshooting), there are two helpful image-processing procedures. A 3 × 3 or higher-order smoothing filter can be applied to the raw images before or after step 1, and/or a threshold can be used below which values are displayed as zero when viewing the FLAP images. Note that smoothing filters other than a block mean may change the intensity value total.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

cDNA constructs of eCFP-β-actin and eYFP-β-actin

Use a pEGFP-β-actin construct (Dunn et al., 2002; *daniel.zicha@cancer.org.uk*) as the basic starting vector. Use enhanced yellow and cyan fluorescent protein coding regions from pEYFP-C1 and pECFP-C1 (Clontech), respectively, to substitute the eGFP coding region using conserved restriction enzyme sites (James Monypenny and Daniel Zicha, Cancer Research UK; *daniel.zicha@cancer.org.uk*). Maintain the 45-bp linker (TCC GGA CTC AGA TCT CGA GCT CAA GCT TGC GGC CGC GCC GCG GCC) between eGFP and β-actin in the pEGFP-β-actin vector in the new fusions. Microinject the DNA in distilled water. The amino acid translation (SGLRSRAQACGRAAA) has net positive charge and 40% hydrophobic residues, ensuring linker flexibility in a cytosolic environment.

Hot wax mixture

Prepare a 1:1:1 (w/w/w) mixture of beeswax, soft yellow paraffin, and paraffin (melting point 46°C) maintained at 54°C in a wax bath.

All of the above materials are available from Fisher.

Optical chambers

Fabricate optical chambers from 76 × 26–mm (as cut) glass microscope slides (Chance Propper), No. 3, 76 × 26–mm glass coverslips, and No. $1\frac{1}{2}$, 18 × 18–mm glass coverslips (Chance Propper). Drill a ~10 mm hole centrally through the glass slide using a diamond-tipped drill (Proxon) under water. Fix a 76 × 26–mm coverslip to one face using Sylgard 184 silicone elastomer (Dow Corning) and allow it to set overnight at room temperature.

While in use, the other face of the medium-filled chamber is sealed with an 18 × 18–mm coverslip carrying the cell culture (see Basic Protocol).

COMMENTARY

Background Information

Fluorescence microscopy has long been the most important tool for revealing the changing distribution of specific proteins within living cells, but it is only recently that fluorescence methods have enabled the study of other aspects of molecular dynamics (see reviews by Lippincott-Schwartz et al., 2001; Holt et al., 2004). One serious limitation of conventional fluorescence localization is that it reveals little of the relocation of molecules during cyclical, steady-state dynamics. Thus critical activities such as the constant depolymerization, diffusive transport, repolymerization, and treadmilling of structural molecules of the cytoskeleton cannot be visualized by this means.

Two methods developed more recently for studying the relocation of molecules, fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP), can have a sufficiently high time resolution to enable diffusion rates of proteins

within the cytoplasm to be estimated (see review by Lippincott-Schwartz et al., 2001). Their chief disadvantage is that the bleached molecules themselves cannot be tracked. On the other hand, fluorescent speckle microscopy (FSM) permits the study of slow molecular movements such as treadmilling and translocation within polymerized structures (*UNIT 4.10*; Waterman-Storer and Salmon, 1997). It relies on a low percentage of tagged molecules (<1%) to produce a discontinuous pattern that can then be used to determine whether the labeled polymer is stationary or translocating. It cannot detect the much more rapid movements of freely diffusing molecules, but this is considered an advantage because it identifies which molecules are polymerized. A refinement of this technique detects the signals from single fluorophores and permits the sites of polymerization and depolymerization of the tagged molecule to be located and their rates to be estimated (Watanabe and Mitchison, 2002). Although complementary to some extent, FRAP, FLIP, and FSM cannot easily provide a full picture of the dynamics within a cyclical polymerization/depolymerization system because the depolymerized molecules cannot be tracked directly.

In contrast, photoactivation of fluorescence (PAF) allows specific molecules to be "flash" labeled at a chosen site and then tracked directly, regardless of whether or not they are polymerized. Earlier PAF methods relied on caged fluorophores and, although they yielded much significant information in the hands of skilled investigators (see review by Mitchison et al., 1998), results were generally disappointing. Uncaging the fluorophore requires exposure to intense UV light, and radiation damage cannot be ruled out. Moreover, molecules tagged with caged fluorophores have to be microinjected or otherwise introduced directly into the cell, and often clump or fail to distribute to their natural locations within the cell. This latter shortcoming led to the development of fluorescent proteins that could be expressed by the cells from cDNA constructs and photoactivated directly. Again, early results were disappointing, and it is only very recently that efficiently photoactivatable fluorescent proteins have been developed (Patterson and Lippincott-Schwartz, 2002; Chudakov et al., 2003). These promise to contribute a great deal to the knowledge of cellular dynamics in the future.

The FLAP method is an alternative approach to the problem of direct and localized photolabeling of a molecular species (Dunn et al., 2002). There is no need to use specialized fluorophores. The principle is simple and relies on photobleaching instead of photoactivation to label the targeted molecules. Suppose that, instead of a single fluorophore, each molecule carries two fluorophores that have differing spectral properties so that they can be imaged separately. If only one of the fluorophores is photobleached, then the resulting molecules are unambiguously labeled: they are the only molecules carrying just one active fluorophore. Although the bleached molecules cannot be imaged directly, one fluorescence image shows where all the molecules are, while the other shows only where the unbleached molecules are. If image intensities have been matched, simply subtracting the second image from the first generates an absolute FLAP image that shows where the bleach-labeled molecules are. Thus FLAP combines the best features of FRAP and PAF. In FRAP, the molecules can no longer be imaged after they have been targeted, whereas in PAF they cannot usually be imaged before they have been targeted. With FLAP, full information is retained on the distributions of both targeted and nontargeted molecules. It is therefore possible to construct other useful virtual images such as relative FLAP, which shows the percentage of bleach-labeled molecules at any given site (Zicha et al., 2003).

It is not an absolute requirement of the FLAP method that a single molecule carry both fluorophores. Indeed, there are some advantages when they are carried by different individual molecules of the same species. In this case, if each colocalizes with the corresponding native molecules as expected, they will still colocalize with each other. However, colocalization now depends on the statistical properties of molecular distribution and, in order to obtain noise-free FLAP images, it is important that both tagged molecules be present in large numbers. This is usually not a problem if the corresponding native molecules are abundant in the cell. It is hard to specify how many molecules need to be carrying fluorophores compared to the number of native molecules, since this depends on many other factors such as detection efficiency, pixel size, and post-processing. A general guideline is that results comparable to PAF or better will be obtained if the two fluorophores give well matched, noise-free images prior to photobleaching. The effect of having the fluorophores on different molecules is, roughly, that the error noise in the final FLAP image is doubled (see Critical Parameters).

The chief competitors to FLAP for flash labeling molecules at specific sites are PAF techniques. Each method has advantages and shortcomings. Although first in the field, the earliest PAF methods using caged fluorophores are probably now the ones with the most disadvantages. First, there are difficulties in conjugating and purifying the protein in sufficient quantity without impairing its activity, as well as in introducing it into cells so that it colocalizes with native protein. A serious limitation is that caged compounds cannot yet be incorporated into cDNA constructs and expressed by the cells. The photoactivatable fluorescent proteins are much more promising in this respect, and recent developments suggest that some will soon be able to be activated and deactivated repeatedly and efficiently (Chudakov et al., 2003), which will be a decided advantage over current FLAP methods.

On the other hand, an interesting feature of the FLAP method as described here is that there is much less need to worry about photodamage occuring to the photolabeled molecules. The method calculates where the virtual bleached molecules are located, i.e., where the bleached molecules would be assuming that they were not damaged, and it is not of much concern what happens to the real bleached molecules. While it is still possible that reactive intermediates generated by photobleaching might cause problems, the compact barrel-like structure of GFP and its variants is thought to shield the external environment from these (Lippincott-Schwartz et al., 2001). Coupled with this, FLAP has the advantages of not requiring specialized fluorophores, and the bleaching process is rapid and efficient using the 514-nm line of a standard argon laser on the LSM 510, whereas photoactivation may require an additional blue diode laser (the alternative two-photon photoactivation is very slow; *UNIT 4.11*). The main advantage of FLAP, however, is that all the tagged molecules are available for imaging throughout the experiment, whether or not they have been photobleached. In the authors' experiments (see Anticipated Results) it has been possible to see throughout where all the actin is, as well as where it has been FLAP-labeled. It may turn out to be possible to do this in conjunction with PAF, but it will require either a second reference fluorophore, as with FLAP, or a second way of exciting the molecules to fluoresce before photoactivating them.

A potential shortcoming of the FLAP method is that colocalization of the two fluorophores is not perfect, but relies on the statistical properties of large numbers of individual molecules. In practice, the authors have not found this to be a problem and have obtained results that compare very favorably with photoactivation methods (but see Critical Parameters). For the future, however, one way around this problem would be to achieve almost perfect colocalization by fusing both fluorophores to the same molecule. This has not been done in this unit because, besides the potential problems of creating a bulkier molecule that may no longer colocalize with corresponding native molecules, it would no longer be possible to ignore any photodamage that might occur to the bleached molecules. Moreover, there is an additional complication that the two fluorophores used in this unit are capable of fluorescence resonance energy transfer (FRET; *UNIT 17.1*) when very close together on the same molecule. Even so, there are early indications that the FRET complication may not be a serious disadvantage and may actually enhance the FLAP signal; however, there are still worries that the FRET efficiency may alter during configuration changes such as polymerization.

Critical Parameters

Expression levels are critical. At high expression levels, there is a danger that FRET may occur in regions of high fluorophore density even when the two fluorophores are carried on separate molecules as described in this unit (see Troubleshooting). On the other hand, the FLAP images would be unusably noisy if the density of fluorescent molecules were very low, as is required by the FSM method. This is especially true when the fluorophores are carried on separate molecules. The binomial theorem can be used to predict these statistical errors in the FLAP signals. Consider first the case in which there happen to be 1000 molecules of CFP-YFP-actin in a pixel and half of them are expected to have a photobleached YFP. Theory predicts that, in repeated experiments, the number of molecules found to be bleached by absolute FLAP would be 500 ± 16 (mean $\pm$ SEM). In the case of relative FLAP, the bleached fraction would be 0.5 ± 0.016. Now, consider the case in which the two fluorophores are not on the same molecule but there still happen to be 2000 fluorophores in the pixel: half are expected to be CFP-actin and half YFP-actin. Half of the latter are expected to have been photobleached. Theory now predicts that the number of molecules found to be bleached by absolute FLAP would be 500 ± 37, and the fraction of YFP-actin molecules

found to be bleached by relative FLAP would be 0.5 ± 0.027. Thus, one can conclude that having the fluorophores on different molecules approximately doubles the error of the FLAP estimates. This also holds true if there are only 200 fluorophores in the pixel, in which case the figures are: 50 ± 5; 0.5 ± 0.05; 50 ± 12; and 0.5 ± 0.09, respectively. The relative errors are proportional to the square of the number of molecules, so it is necessary to express four times as many fluorescent molecules in order to halve the relative error.

A further requirement, as with most fluorescence microscopy, is that the tagged molecules accurately colocalize with native molecules of the same species. There is evidence that GFP variants, fused to actin in the configuration used by the authors, do this well (Choidas et al., 1998). Ensuring that the two different variants are incorporated into otherwise identical cDNA constructs will give the best colocalization for FLAP even when colocalization with the native species is slightly impaired.

Even the best apochromatic objectives show a significant lateral and axial chromatic aberration. This means that the CFP and YFP images may not exactly coincide both laterally and vertically, and it is necessary for good results that the cell be close to the optical axis of the objective (see Troubleshooting) and that there be no drift in focus. Temperature stabilization is essential for maintaining a fixed focal plane. In the past, the authors have obtained satisfactory results using a fan and temperature-controlled heater within a spacious microscope dust cover placed over the entire microscope. However, a properly constructed air-flow chamber (Fig. 21.2.1) is essential for consistently good results. The thermocouple is placed very close to, but not touching, the optical chamber (see Troubleshooting). The temperature controller is

Table 21.2.1 Troubleshooting Guide to FLAP

Problem	Possible cause	Solution
Regions of cell edge show in FLAP images even before bleaching	Chromatic aberration	Minimize by using fluorescent beads to find best region of objective.
Rhythmic noise or parallel lines	Vibration from heater, thermocouple, table, or computer	Eliminate fan/computer vibration; make sure that table is properly isolated.
Focus drift	Temperature fluctuation	Check heater stability and presence of drafts.
Artifacts on top 1-2 lines of first image scanned after a frame interval	Software bug in acousto-optic driver	Avoid frame intervals if possible. Avoid placing critical part of cell near top of image.
Difference signal is not zero everywhere before bleach	Images not matched	See Basic Protocol, step 14 or match images during post-processing.
	Fluorophores not colocalized	Choose new fluorophore(s).
Striped or fluctuating phase image	Polarizing filter in the light path	Remove polarizing filter from the light path.
Lower (negative) FLAP signal in areas of dense expression before bleach	FRET	If unacceptable, use shorter expression times, compensate during post-processing, or choose new fluorophore(s).
Noisy fluorescence images on one or both channels.	Low expression	Wait longer for expression. Adjust cDNA injection ratio.
	Too much gain in photodetector amplifier	Increase laser power and reduce gain (but keep fade rates matched if possible).
	Too much magnification (pixels too small)	Use lower zoom factor or average more frames.

trained to maintain a temperature of 37°C, and should do so, ideally, within a range of ± 0.1°C.

Troubleshooting

See Table 21.2.1 for a troubleshooting guide.

Anticipated Results

FLAP has been used to estimate the diffusion rate of G-actin in cytoplasm (Zicha et al., 2003), and the result agrees well with previous estimates using FRAP (McGrath et al., 1998). Relative FLAP has proven particularly useful for detecting the rapid transport of actin to the cell's leading edge from regions up to 12 μm behind the edge (Zicha et al., 2003). Four FLAP images showing some of the results obtained from the cell featured in Figures 21.2.2 and 21.2.3 are shown in Figure 21.2.7. Note that the absolute FLAP images (Fig. 21.2.7A and C) show the highest intensity in parts of the bleach region where the density of actin is greatest (see Fig. 21.2.3), whereas relative FLAP (Figure 21.2.7B and D) gives a more uniform signal throughout the bleach region. In the later relative FLAP image which started 3.9 sec after bleaching (Figure 21.2.7D) highly labeled actin (∼60%) can be seen at small regions of the cell's leading edge. This phenomenon is not noticeable in the absolute FLAP image (Figure 21.2.7C) because these thin marginal regions have a relatively low density of actin. When interpreting the FLAP images, bear in mind that the top of the image has been scanned first and that there can be a time difference of several seconds between the top and bottom of the image. It is especially important to compensate for the scanning time when fitting mathematical models of fast molecular processes such as diffusion of monomer (see Internet Resources).

Time Considerations

Cell culture

Plan ahead by at least 72 hr to allow for sufficient cell density, depending on growth rate of cell type.

Microinjection

This should take no more than 15 min if injecting cells at room temperature without CO_2.

Expression of constructs

Allow at least $2\frac{1}{2}$ hr for expression and maturation of GFP and variants.

Filming chamber preparation

Chambers should be prepared well in advance (usually left overnight) and kept sterile in a sealed container until needed.

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Zicha, D., Dobbie, I.M., Holt, MR., Monypenny J., Soong, D.Y.H., Gray, C., and Dunn, G.A. 2003. Rapid actin transport during cell protrusion. *Science* 300:142-145.

Key References

Dunn et al., 2002. See above.

First description of the technique.

Zicha et al., 2003. See above.

Describes an application of the technique.

Internet Resources

http://www.lis.ch

Web site of Life Imaging Services, which supplies microscope temperature control systems.

http://www.zeiss.com/us/micro/home.nsf/Contents-FrameDHTML/286BA4D22B14DEE985256B4A007C3686

Zeiss Web site from which LSM Reader can be downloaded.

http://www.sciencemag.org/cgi/content/full/300/5616/142/DC1

Supplementary online material for Zicha et al. (2003). Describes diffusion modeling.

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