

Using Cell-ID 1.4 with R for Microscope-Based Cytometry

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ABSTRACT

This unit describes a method for quantifying various cellular parameters (e.g., volume, total and subcellular fluorescence localization) from sets of microscope images of individual cells. It includes procedures for tracking cells over time. One purposefully defocused transmission image (sometimes referred to as bright-field or BF) is acquired to locate each cell. Fluorescent images (one for each of the color channels to be analyzed) are then acquired by conventional wide-field epifluorescence or confocal microscopy. This method uses the image processing capabilities of Cell-ID (Gordon et al., 2007) and data analysis by the statistical programming framework R (R-Development-Team, 2008), which we have supplemented with a package tailored to analyze Cell-ID output. Both programs are open-source software packages. *Curr. Protoc. Mol. Biol.* 84:14.18.1-14.18.27. © 2008 by John Wiley & Sons, Inc.

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INTRODUCTION

This unit describes a method for quantifying various cellular parameters from individual cells using sets of microscope images. It includes procedures for tracking cells over time. Using this method, the user can measure total and subcellular localization (nuclear, plasma membrane) of fluorescence for multiple fluorescence color channels (Colman-Lerner et al., 2005; Yu et al., 2008). This method uses the image processing capabilities of Cell-ID (Gordon et al., 2007) and data analysis by the statistical programming framework R, both open-source software packages. The first step for successful cytometry entails acquiring at least one set of images for each field of cells. Each set is composed of one purposefully defocused transmission image (referred to as bright-field, or BF) that will be used to locate each cell and one fluorescent image for each of the color channels to be analyzed (x FP, where x designates the color of the fluorescent protein). Fluorescence images may be acquired by conventional wide-field epifluorescence or confocal microscopy. Cell-ID processes the images and outputs annotated TIFF images and a tab-delimited file with information extracted from each cell, for each time point and each fluorescence channel. Finally, the user analyzes the data using R (R-Development-Team, 2008), which is supplemented with a package tailored to analyze Cell-ID output.

Like the original Cell-ID 1.0 (Gordon et al., 2007), Cell-ID version 1.4 supports a full command-line interface, which allows sophisticated automatic analyses using scripts; it can be compiled to run on most computer systems. Cell-ID 1.4 incorporates a graphical user interface (VCell-0.1), which provides easy testing options that help the user choose the correct parameters to process the experiment in batch mode, as well as bug fixes and

improvements. Although Cell-ID was originally tailored for yeast cells, it works well with nonadhering mammalian cells (Gordon et al., 2007).

R is an environment for statistical analysis, data manipulation, calculation, and graphical display (<http://www.r-project.org>). Rcell is a package that was created to aid in the analysis of the Cell-ID output files. Rcell contains a set of functions for loading these files to R, filtering out unwanted cells, displaying images, and plotting simple and compound variables.

This unit focuses on procedures for measuring fluorescent protein–based reporters. However, nearly identical approaches can be used when measuring signals from fluorescent antibodies or light-emitting enzymes. The Basic Protocol presents the methodology used to acquire, process, and analyze quantitative single-cell data extracted from time-course experiments. The Alternate Protocol describes a procedure for quantifying measurements obtained in intensity-based fluorescence resonance energy transfer (FRET) experiments. Support Protocol 1 provides information about obtaining and installing necessary software. Support Protocol 2 presents a short guide for preparing yeast and mammalian cells for imaging. Support Protocol 3 describes how to perform FRET calculations using split images and how to measure FRET in the nucleus and plasma membrane of yeast.

BASIC PROTOCOL

EXTRACTING QUANTITATIVE INFORMATION FROM SINGLE CELLS

In this protocol, Cell-ID and R are employed to process and analyze cell images in time-course experiments, where the same cells at one or more positions (xy coordinates in the stage corresponding to a field of cells) are followed over time. For each time point, the user will acquire one set of images. The simplest case is when one has only one time point; in this case, the user collects only one image set per position. If the cells at the different time points are not the same (e.g., when taking different samples from a culture at different times) each set should be labeled with a different position number (see below for how to label files), since in this type of experiment one will not be following the same cells over time, but rather different cells from the same population over time.

The combination of Cell-ID and R is most powerful when following the same cells in time series, since it allows the user to measure dynamic processes in living cells, e.g., reporter protein expression, relocalization of proteins in response to specific signals, correlations between fluorescent protein levels in single cells, changes in protein conformation and oligomerization state by FRET (fluorescence resonance energy transfer; see Alternate Protocol), and protein and mRNA degradation rates or growth rates (Colman-Lerner et al., 2005; Gordon et al., 2007; Yu et al., 2008). Time series are especially useful for measuring small signals close to the levels of noise, which are best detected as changes over time.

Materials

- Cells of interest affixed to the bottom of multiwell glass-bottom plates or on slides (Support Protocol 2)
- Optically appropriate support for cells (multiwell plates preferred over slides and cover slips for easy access)
 - For oil immersion with high-NA objectives: glass-bottom, coverslip-thin 96- or 384-well plates (available from Arctic White)
 - For air or water immersion objectives: plastic multiwell plates

Hardware

- Fluorescence microscope with the following features:
 - Inverted (to allow access to live cells during imaging over time), preferable
 - Stage with motorized z control, preferable to manual focus for convenience and reproducibility
 - Stage with motorized xy control (for live imaging of multiple fields of view, e.g., in neighboring wells with cells treated differently), optional

Motorized shutter (for precisely controlling exposure time of fluorescence illumination), optional for bright-field illumination
High-numerical-aperture (NA; e.g., >1.2 for 60× objective), chromatically corrected (at least PlanApo) objective to capture as much light as possible, especially important for work that requires subcellular colocalization
Black-and-white (not color for quantitative imaging), cooled CCD camera (when using non-confocal microscope)
Appropriate filter cubes or wheels (motorized *z* position optional for complete automation of imaging)
PC to run microscope
Acquisition software, e.g., Metamorph (Molecular Devices), ImagePro (Media Cybernetics), or μ manager (open-source microscopy software initiative at UCSF; <http://www.micro-manager.org>)
PC workstation with UNIX, LINUX, or Windows XP or higher operating systems; or Apple workstation with Mac OS X operating systems and X11 installed

Software

Cell-ID (Gordon et al., 2007) and Vcell-ID
R (R-Development-Team, 2008) and the package Rcell (see Support Protocol 1)

Image files

Images in TIFF format (gray-level TIFF files of 8 and 16 bits); time-related information can be extracted from the Metamorph-generated image files (see below)

Determine experimental settings

1. Determine the correct magnification for your application.

One can normally use a 60× oil-immersion objective for yeast and a 20× air objective for mammalian cells. The advantage of 20× magnification is that each image contains many more cells, and the air objective makes glass-bottom plates unnecessary; the disadvantage is that the objective captures less light and produces images with less spatial resolution.

2. Prepare cells with the lowest expected fluorescence levels for each channel (e.g., cells not expressing any fluorophore) and cells with the maximum levels for a given experiment (e.g., fully-induced cells when using fluorescent protein-based transcriptional reporters).
3. Place these cells, either affixed to the bottom of multiwell glass-bottom plates or on slides, in the microscope and find the focus using one of the fluorescence channels.
4. At this *z* position, move the stage to a fresh unexposed field of cells and, starting with the lowest energy (longest wavelength) channel, acquire a series of images with increasing exposure times. Determine which is the lowest exposure time necessary to visualize the cells that do not express fluorescent protein.
5. Repeat step 4 using cells expressing the reporter in the appropriate fluorescence channel, with different exposure times, until pixel intensities become saturated.

*In many applications (e.g., ImageJ and Metamorph), moving the cursor over an image will display the *xy* coordinates and the intensity of the pixel. Values for saturated pixels depend on the image depth: 255 for 8-bit and 65535 for 16-bit images.*

The times determined in steps 4 and 5 are the minimum and maximum exposure times. In some cases, the exposure time determined in step 4 is higher than that of step 5. In this case, you need a CCD camera with larger dynamic range, or you will have to change exposure time during the experiment (this option will complicate analysis).

Exposure time is a critical parameter. If exposure is high, sample photobleaching and phototoxicity increase. This might be an issue when performing a time-course experiment. On the other hand, if exposure is too low, the signal-to-noise ratio might be too low. Choose an exposure time appropriate for the particular experiment.

Acquire images

Steps 6 and 7 represent images from one set of images, for one position and one time point.

6. Acquire the bright-field image:
 - a. Take an image with the cells at the focal plane using transmitted light.
 - b. Move the z knob so that the objective moves slightly down (away from the stage) until a dark ring surrounds the cells (Fig. 14.18.1).
 - c. Record how much the stage was moved.

For a 60 \times objective, this is usually 100 to 200 nm.

- d. Take another image.

This is the bright-field image that Cell-ID uses to find the cells.

- e. Label the image BF_Position [X] .tif, where [X] is a number. Make sure all of numbers [X] have the same number of digits, e.g., label images between 1 and 99 as 01, 02, 03, and so on; label images between 1 and 999 as 001,002,003 and so on. If following the *same* cells over time and taking images for multiple time points per position, name the file BF_Position [X] _time [Y] .tif; in this case, also make sure that the numbers [Y] have the same number of digits.

To be able to find the cells with Cell-ID, a bright-field image is required, which can be obtained with a transmission channel in the confocal setup. The images obtained from this channel usually have more noise than those obtained with a CCD camera. This is due to the fact that the integration time for each pixel is much lower in a confocal image, thus increasing the relative shot noise. Because Cell-ID was originally designed for CCD images, it has a reduced performance for these noisy images. To overcome this limitation, an average (or low pass filter) of several consecutively obtained transmission images can

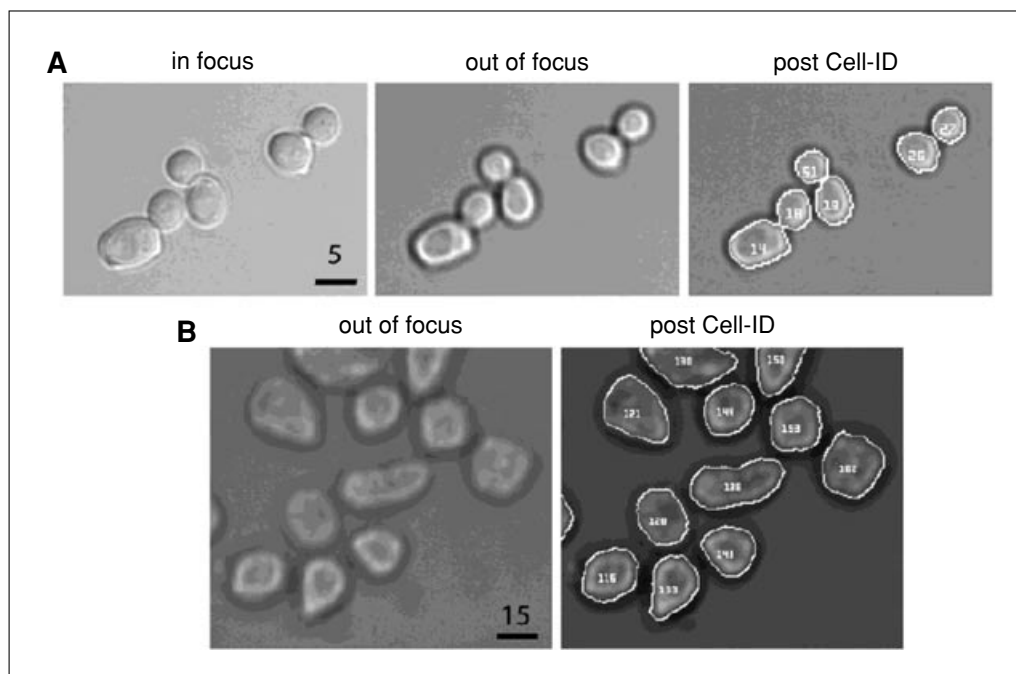


Figure 14.18.1 Examples of yeast and mammalian cells processed by Cell-ID. **(A)** From left to right: yeast in focus, slightly defocused (note the dark ring on the border of the cells), and the same cells after Cell-ID has identified each one and traced their borders correctly. Bar = 5 μ m. Magnification: 60 \times . **(B)** HEK293 cells fixed, trypsinized, and imaged (see Support Protocol 2 and the Basic Protocol), before (left) and after (right) Cell-ID has located them in the image. Bar = 15 μ m. Magnification: 20 \times .

be created. Doing this will average out the noise and help to reduce the amount of spurious and badly found cells when processing the images. The averaging can be done either with the image acquisition software used (e.g., using a Kalman filter), or as a preprocessing step before executing Cell-ID.

7. Acquire fluorescence images:
 - a. Move the *z* position again to the focal plane (up the same distance you moved it down).
 - b. Acquire one fluorescence image for each channel required. Using the settings determined in steps 4 and 5, take images from lowest to highest energy (from red to violet) to minimize cross-photobleaching of the sample.
 - c. Label images according to the color, in a stereotyped way, as in step 6. For example, RFP_Position01_time001.tif, YFP_Position01_time001.tif, and CFP_Position01_time001.tif.
8. Repeat steps 6 and 7 for as many positions and time points as needed. Number each image set consecutively.

Acquiring images with a confocal microscope has several advantages over epifluorescence. In first place, it eliminates out-of-focus light and has better spatial resolution (i.e., smaller point spread function). These assets could be useful for the quantification of fluorescence in subcellular compartments. In addition, if the microscope is equipped with an optoacoustic modulator, the illumination intensity can be fine-tuned with great precision, allowing the optimization of the fluorescence signal while maintaining acceptable photobleaching levels.

9. *Optional:* If you want Cell-ID to subtract a background image for every fluorescent color, then take an image with the shutter closed with the appropriate exposure time determined for each color in steps 4 to 6. Label these images appropriately, e.g., YFP-dark.tif, CFP-dark.tif, and RFP-dark.tif for YFP, CFP, and RFP image exposure times, respectively.
10. *Optional:* If you want Cell-ID to correct for uneven illumination, take images with each filter cube and exposure settings in areas with medium but without cells. Label these images, e.g., YFP-flat.tif, CFP-flat.tif, and RFP-flat.tif.

Usually rich culture medium (e.g., YPD) is autofluorescent enough to give a large enough signal for Cell-ID to calculate a correction. If the illumination is uneven, the fluorescence emitted will be, too, and Cell-ID will attempt to apply a flattening correction based on these images.

*Additionally, if the medium is significantly autofluorescent, care should be taken to avoid *xy* positions that are near the edges of the wells.*

11. Place all images in the same folder.

Process images with Cell-ID

12. Start a Vcell-ID session. PC users can click on the VCell-ID icon. For Linux and Macintosh users, start VCell-ID from an X11 terminal, typing at the prompt: `vcellid &`. The VCell-ID window will appear. The main window has several tab buttons across the menu.
13. Select Setup→Load Images. The Load Images window will appear (Fig. 14.18.2).
 - a. In the drop-down menu below “path,” select the folder containing the images. If all images are not in the same location, deselect “force same path,” and then select the appropriate path for each image type.

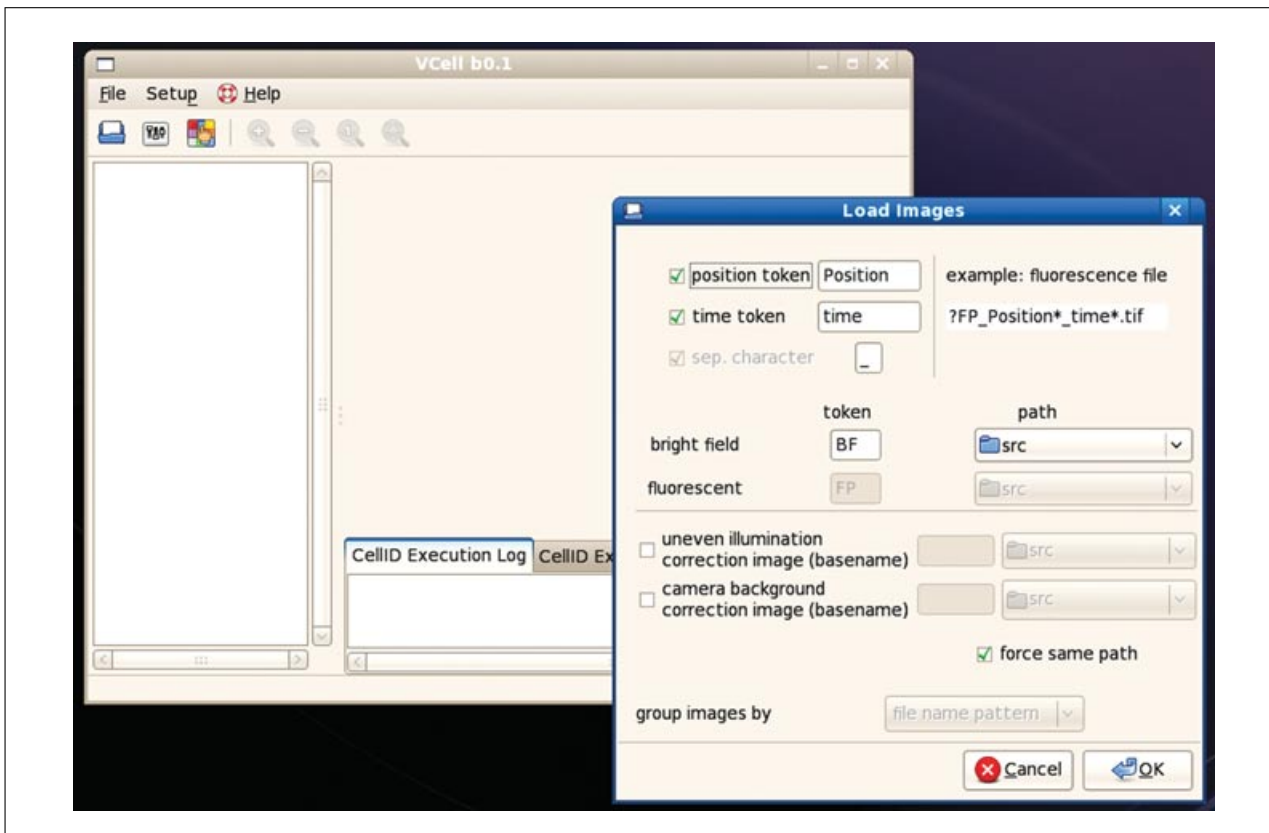


Figure 14.18.2 The main VCell window with the Load Images window open, before selecting the folder containing the images.

- b. In the drop-down menu next to “group images by,” select how Cell-ID should map bright-field images to fluorescence images. Select Metamorph if Metamorph was used to acquire the images. Otherwise, select “file name pattern.”

With the Metamorph option, Cell-ID will use the bright-field image closest in time to a given fluorescence image based on its internal TIME TAG (embedded in each Metamorph TIFF image, not the file creation time shown by the computer). Otherwise Cell-ID will use the bright-field image closest in time to a given fluorescence image based on its NAME. For example BF.Position03_time005.tif will be used to process YFP.Position03_time005.tif.

- c. For images not named as suggested in steps 6 and 7, change the “position” and “time” token, and the “separator character” to match those of the images.
 - d. For an experiment without time points, uncheck “time token.”
 - e. For dark and flat images, include the tokens “camera background correction image” and “uneven illumination correction image” (see examples in steps 9 and 10).
 - f. Click OK to load the images and to close this window.
14. Use the experiment tree shown on the left panel in the main window to explore the images in your experiment. The experiment is now organized based on positions. Click on an image file to load it on the central window. Now you can zoom in or out.
 15. Select Setup→Image Setup. The Image Setup window will appear (Fig. 14.18.3).

- a. For bright-field images to be treated as fluorescence images, check “bf as fl.”

This is sometimes useful to filter out out-of-focus cells, since these cells appear brighter in the bright-field image.

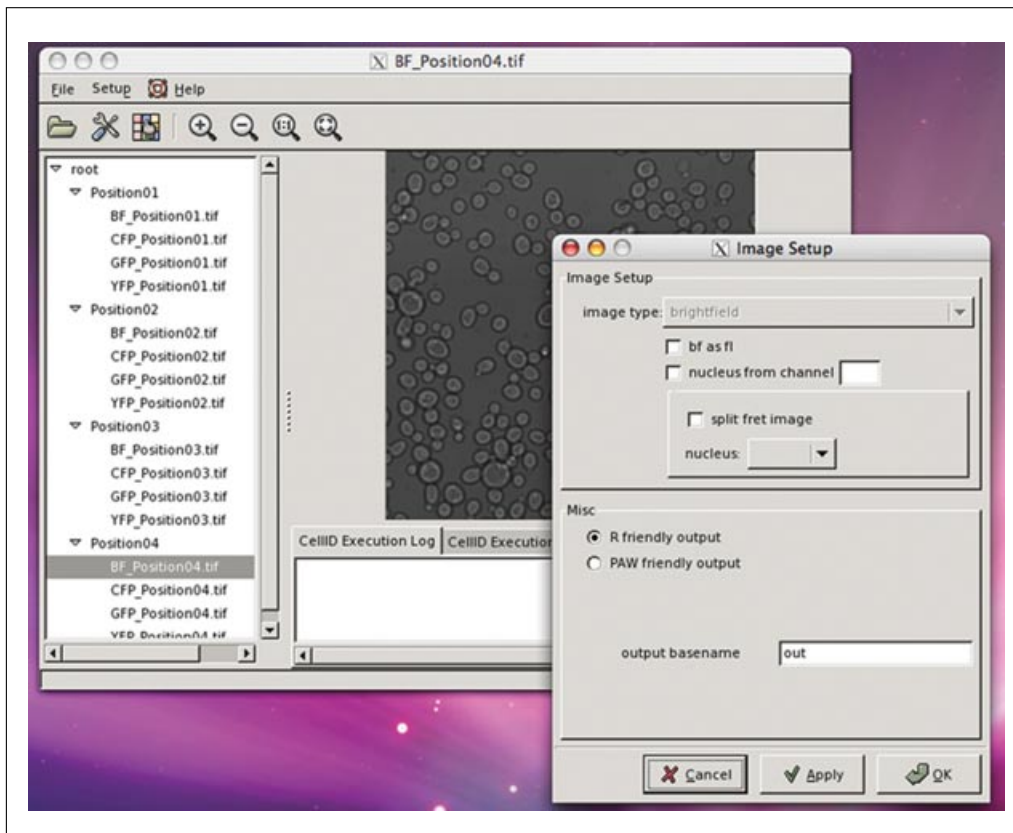


Figure 14.18.3 The main Vcell window with the Image Setup window open, after applying the settings shown in Figure 14.18.2. On the main window at the left, is the directory tree with BF_Position04.tif selected. That image is shown in the central window.

- b. To find fluorescently labeled nuclei with Cell-ID, check “nucleus from channel” and type the image token corresponding to it, e.g., YFP.
- c. To analyze a split image FRET experiment, check “split fret image” and proceed to the Alternate Protocol.
- d. To analyze the data with PAW (<http://www.cern.ch/paw>), select “PAW friendly output.”

PAW (Physics Analysis Workstation) is an interactive system based on several components of the CERN Program Library. It provides a set of commands acting on specific objects, e.g., histograms, event files (Ntuples), vectors, and was conceived as an instrument to assist researchers in the analysis and presentation of their data.

- e. Type the string you want Cell-ID to add to the name of the output data file (default is “out”).
16. Select one bright field image on the tree and right click (option-click in Mac’s X11). Select Show Test Dialog on the pop-up menu. The segmentation window will appear.

The choice of values in this window will profoundly affect how Cell-ID finds cells.

Set the following parameters:

- “max dist over waist” (D/W)
- “max split over minor”

These two parameters were designed to determine if two neighboring cells are actually two cells or a single, budding yeast cell. The defaults here work well with yeast. If the cell’s perimeter divided by the minimum distance between any two points of the cell boundary (waist) is less than max.dist.over.waist, Cell ID will try splitting them.

The split will be done only if the waist of the original cell (i.e., the minor distance between any two points of the cell boundary) divided by the minor axis of both new cells is less than the value for this parameter.

“min pixels per cell”

“max pixels per cell”

The minimum and maximum number of pixels that a cell needs to have to be considered a cell by Cell-ID is useful for limiting the number of spurious cells located by Cell-ID. The actual number depends on the magnification of the objective and the size of pixels in the chip of the CCD camera. For example, in images taken with an oil-immersion 60× objective on a CCD camera with 13- μm pixels, exponentially growing yeast have an average area of 400 pixels, and limits between 200 and 600 work well to include all cells.

“background reject factor”

The code makes an initial decision about the intensity of the boundary pixels. To do this, it takes the mean intensity of the bright-field images and subtracts a set number of standard deviations. It then starts by considering all intensities below this value as being parts of the cell borders. The number of subtracted standard deviations is the parameter “background reject factor.” Usual values are between 0.4 and 1.2.

Bright-field images taken slightly out of focus may do better with higher values (i.e., higher values will better avoid spurious cells), but if the cell boundaries in the image are too narrow, a smaller value may be necessary, which might have the consequence of increasing the level of incorrectly called cells.

“Tracking comparison”

Cell-ID attempts to track cells over time. The value of this parameter is the minimal fractional overlap between two cells in consecutive time points for them to be considered the same cell. The default value is 0.2.

“Frame alignment” and “single cell alignment.” Leave these options on their default values.

Cell-ID by default will find the best whole-frame overlap between the bright-field and each of the fluorescence images, compensating for small frame shifts between images. Similarly, Cell-ID will move the contour of each cell found on the bright-field image to match the cells in the fluorescence images. This feature is useful when cells move slightly between image acquisitions.

17. After selecting values for these options, click Test Cell-ID. Cell-ID will do a full processing of the whole set corresponding to that bright-field image, and it will display the output files on the main screen (Fig. 14.18.4). It will also create an `out.all.txt` file with the numerical data to be analyzed (see step 15e). Click on OK to return to the main menu.

18. In the bright-field image, check if Cell-ID appropriately found the majority of the cells.

Depending on how clean the background of the bright-field image is, Cell-ID might also have found structures in the image that were “mistaken” as cells. These incorrectly found “spurious cells” can be eliminated later during analysis by appropriately applying filters (see “cuts” below). If Cell-ID did not find the cells appropriately, you will have to troubleshoot it. Changing parameters in the Segmentation Setup window might help (Fig. 14.18.5).

19. Check that the cells in the corresponding fluorescence images have been marked correctly.

20. In time course experiments, test a whole position by clicking on the appropriate folder, followed by Test.

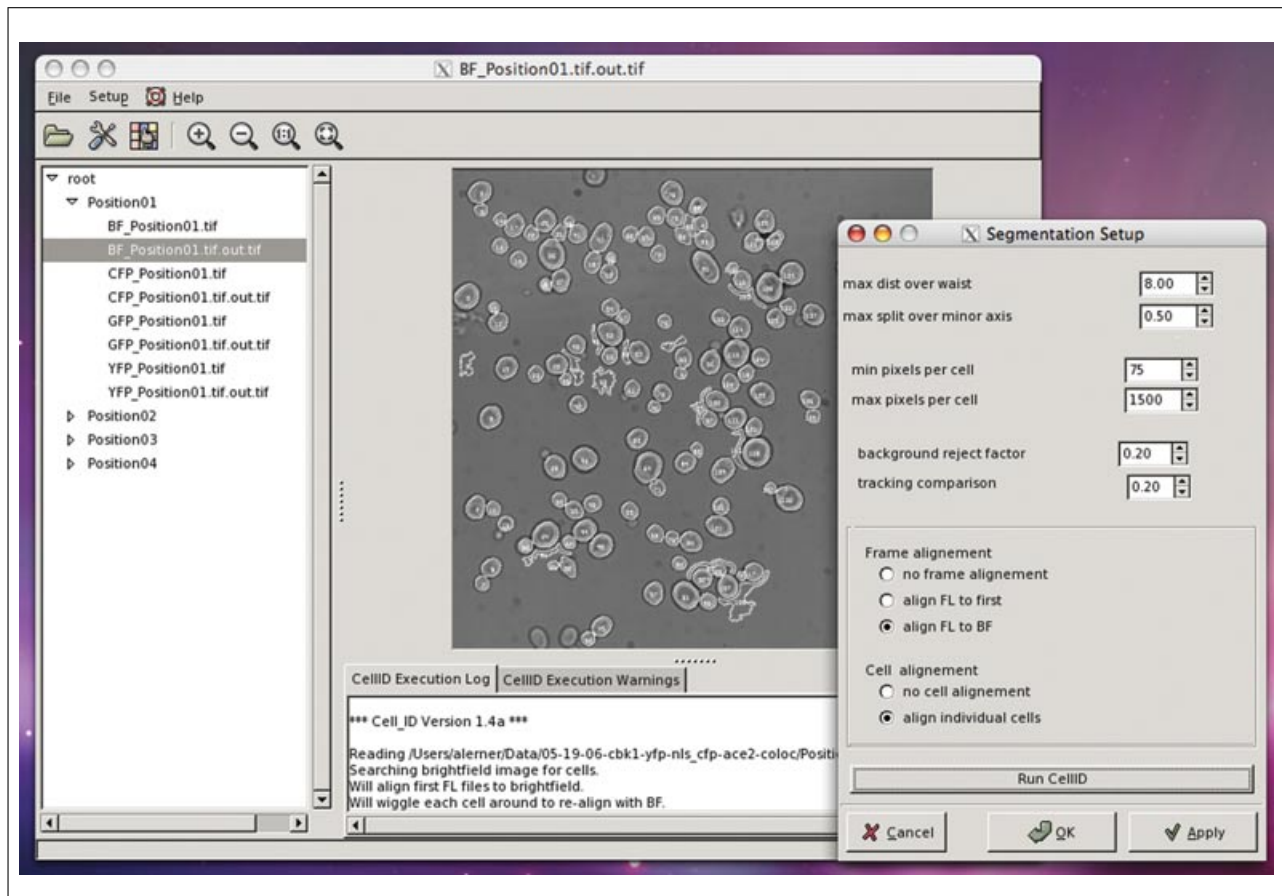


Figure 14.18.4 The main Vcell window with Segmentation Setup open, after running Cell-ID with the parameters shown. Note that on the tree directory there are new images, the `.out.tif` files created by Cell-ID. On the central window the cells have been found by Cell-ID. Note the presence of several structures in the background wrongly identified by Cell-ID as cells.

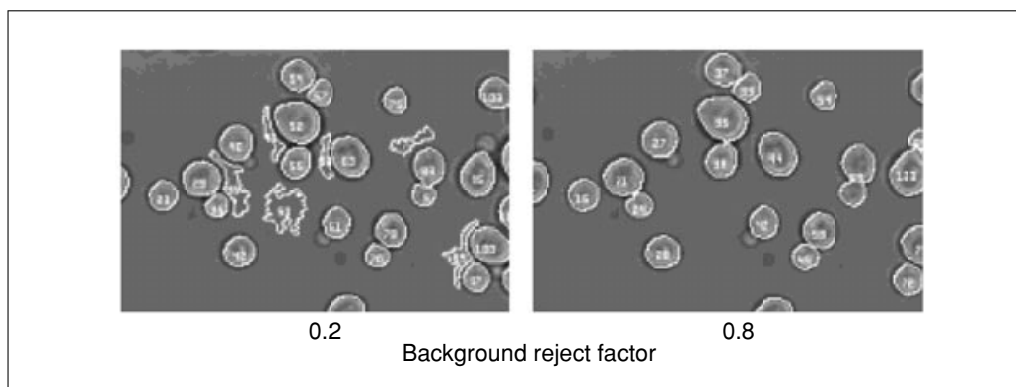


Figure 14.18.5 The effect of changing the value of “background reject factor.” The same image was processed by Cell-ID using two different values for this variable, 0.2 and 0.8 (left and right, respectively). Note that with 0.8 fewer spurious cells were found.

21. If the test was satisfactory, try it on another set.
22. When Cell-ID is finding the cells correctly, right click on the tree view “root” node, and select Show Run Dialog. If not already visible, the Segmentation Run Dialog will appear. Click on the Run button to process the whole experiment. Cell-ID will create new images named identically with the added “out” label before the file extension (e.g., the processed version of `BF_Position01_time01.tif` will be called `BF_Position01_time01.tif.out.tif`). In addition, new folders will also be created with the input files and the generated output files.

Table 14.18.1 Cell-ID Output Variables

Variable name	Description
<i>General measurements</i>	
1 cellID	Cell identification number. In FRET experiments the cells in the upper and lower part of the split image are differentiated by an offset added to this value.
2 t.frame	Time frame of the cell (0 through $n - 1$ where $n =$ number of points in the time course). Not every cell is necessarily found in every time point.
3 time	Time of that time frame in seconds. The time unit is an absolute number of seconds from some time in the distance past, but the time elapsed between time frames is more meaningful (only meaningful if using Metamorph images).
4 xpos	x coordinate of the centroid of the cell
5 ypos	y coordinate of the centroid of the cell
6 f.tot	Sum of the fluorescence image for all the pixels found in that cell
7 a.tot	Area of the cell in pixels
8 fft.stat	Statistic derived from the one-dimensional fast-fourier-transform (FFT) of the function: radius vs. angle, where the radius is the distance from the cell centroid to the boundary at a given angle. Its value is the root of the squared sum of the ratio $\text{FFT}(\omega)/\text{FFT}(0)$ over all $\omega > 0$; for a perfect circle FFT_stat is 0, and we interpret this statistic as a measure of circularity.
9 perim	Circumference of the cell in pixel units
10 maj.axis	Length of the major axis in pixel units
11 min.axis	Length of the minor in pixel units
12 flag	Indicates the image type. For example, for YFP and CFP images, all YFP images would be flag 0 and all CFP would be flag 1, assuming that the YFP image was taken earliest in time
13 rot.vol	Volume of rotation of the cell around its major axis
14 con.vol	Volume of the cell as determined by the conical volume method (Gordon et al., 2007)
15 a.vacuole	Vacuole area: calculated from the region inside the cell that is less brightly fluorescent. In exponentially growing cells expressing fluorescence localized to the cytoplasm, this “dark” region corresponds to the vacuole.
16 f.vacuole	Vacuole fluorescence: calculated from the region inside the cell that is less brightly fluorescent. In exponentially growing cells expressing fluorescence localized to the cytoplasm, this “dark” region corresponds to the vacuole.
17 f.bg	Fluorescence background level; the mode of the distribution of all fluorescence pixels not associated with any cell
<i>To calculate membrane proximal fluorescence (for relocalization experiments)</i>	
18 f.tot.p1	Fluorescence and area of all the pixels interior to the boundary that is one pixel wider than the cell boundary. Numbers thus include the original cell plus an annular region one pixel around the outside of the cell.
19 a.tot.p1	Area of all the pixels interior to the boundary that is one pixel wider than the cell boundary. Numbers thus include the original cell plus an annular region one pixel around the outside of the cell.
20 f.tot.m1	Fluorescence of all pixels interior to the boundary that is one pixel smaller than the cell boundary

continued

Table 14.18.1 Cell-ID Output Variables, *continued*

Variable name	Description
21 a.tot.m1	Area of all pixels interior to the boundary that is one pixel smaller than the cell boundary
22 f.tot.m2	Fluorescence of all pixels interior to the boundary that is two pixels smaller than the cell boundary
23 a.tot.m2	Area of all pixels interior to the boundary that is two pixels smaller than the cell boundary
24 f.tot.m3	Fluorescence of all pixels interior to the boundary that is three pixels smaller than the cell boundary
<i>Information obtained from the “nuclear image” type (Variables contain the area and fluorescence of concentric disks of user-defined radius.)</i>	
25 f.nucl	Total fluorescence in the found nucleus. To find the nucleus, Cell-ID moves a disc with a radius of two pixels around the interior of the cell and finds the location where the disc has the maximum total fluorescence. From that location it calculates the fluorescence within a circle of four pixels of radius. This process is done for every fluorescence image. If some pixels of the disc fall outside the cell boundary, they are not used in the quantification.
26 a.nucl	Area of the found nucleus
27 f.nucl1 to f.nucl6	Same as f.nucl, but using a disc of increasing radius to calculate the fluorescence for each image. f.nucl1 uses a disc of 2 pixels of radius, f.nucl2 uses a disc of 3 pixels, and so forth up to f.nucl6 which uses a disc of 7 pixels of radius.
28 a.nucl1 to a.nucl6	The area corresponding to f.nucl1 to f.nucl6
29 f.nucl7 a.nucl7	Fluorescence and area of all the pixels of the cell interior
30 f.nucl.tag1 to f.nucl.tag7	Same as f.nucl1 to f.nucl7, but the nucleus center (or Gaussian fit) is calculated from the nuclear tagged fluorescent channel, specified in step 15b. If no nuclear channel is specified, these variables are equal to f.nucl1 to 8.
<i>More background information</i>	
31 f.local.bg	Measure of the background level at pixels located 5 radial pixels further out than the cell boundary; thus, a measure of the local fluorescence background level, the average fluorescence per pixel. Only pixels along the annular boundary NOT associated with ANY cell are included; background level here is the mean of the pixels.
32 a.local.bg	The number of pixels used in the background calculation for local.bg
33 a.local	Total number of pixels along the annular region, including all pixels (i.e., pixels associated with cells and pixels not associated with any cell)
34 f.local2.bg	Same information as the previous three rows, but using the
35 a.local2.bg	background level at pixels located x radial pixels outward of the cell
36 a.local2	boundary, where x is one half of the minor axis of the cell.
<i>More volume measurements</i>	
37 a.surf	Surface area as calculated by the union of spheres method (Gordon et al., 2007)
38 sphere.vol	Volume a measured by the union of spheres method (Gordon et al., 2007)

Analyze data with R

Cell-ID will have created one folder named `Position[X]` for each position in the experiment, and within each of these folders it will have created a file with all the data called `out_all`. In this TAB delimited file, the columns contain the variables and statistics calculated by Cell-ID (Table 14.18.1). You can use the program of your choice to analyze it. The following presents an explanation of how to use R with the package for Cell-ID, `Rcell`.

23. Open R and load the R package `Rcell` by typing `require('Rcell')` in the R console, or selecting from the “packages -> load package” menu if using an R-GUI.
24. Type `setwd('c:/MyData/ExpFolder')` in the console, using the path to the directory that contains the Cell-ID output folders, usually the same folder where you stored the images. This will set the working directory.

Alternatively you can do it from the file->change dir menu (in Windows and OS X).

25. Open the Cell-ID output files with the command `X<-load.cellID.data()`. This will load the `out_all` files and restructure the data for efficient filtering and plotting. The function returns an R list object with all the required information associated with the experiment.

All the functions included in the package operate over this object, and its components should not be modified directly, but through the provided functions. You can name it as you prefer; throughout this example, it is called X.

The restructuring of the data modifies Cell-ID output variables (Table 14.18.1) and creates a few extra variables (Table 14.18.2). It appends a channel dependent postfix (the shortest unambiguous string from the channel token, in lower case). For example, if one has YFP and CFP channels, the total fluorescence for each channel becomes `f.tot.y` and `f.tot.c`, respectively. Analogously, `f.bg` becomes `f.bg.y` and `f.bg.c`, `f.nucl` becomes `f.nucl.y` and `f.nucl.c`, etc. Nonfluorescence-related variables are not renamed. For example, `a.tot`, `pos` and `t.frame` remain as such (Table 14.18.1). Type `print.channels(X)` in the console to get a table with the assigned postfix for each channel.

The R commands entered in the console window for data loading might look like this:

```
#Load the R package for Cell-Id.
require('Rcell')
#Set the working directory.
setwd('C:/Path/.../MyData')
#Load the data for all the positions.
X<-load.cellID.data()
#Check for the existing channels.
print.channels(X)
```

Perform quality control

The algorithm used by Cell-ID to find the cells can occasionally make mistakes in the assignation of the cell boundaries and produce badly found and spurious cells (i.e., image structures erroneously scored as cells). One can minimize these errors by adjusting the program parameters (step 16), but usually some badly found cells remain. Furthermore, the program cannot discriminate out-of-focus and dead cells.

26. Filter out unwanted cells from the data set using the R package.

This first filtering step is intended to eliminate from the dataset, spurious, out-of-focus and dead cells. If analyzing a time course, it filters the same cell in different time points independently, reflecting the fact that a cell can move out of focus, get its boundary found differently, or even die between time points. You can, however, require that a given cell appear in a particular set of time points, e.g., all time points after the first five (step 27).

Table 14.18.2 Variables Calculated from and Appended to Cell-ID Output by the Rcell Package

Column number	Variable name	Description
1	<code>pos</code>	Position number (i.e., id of image field); same for a single cell through every set of images of a time course
2	<code>f.m0</code>	Fluorescence of all pixels that are the same as the boundary
3	<code>a.m0</code>	Area of all pixels that are the same as the boundary
4	<code>f.m1</code>	Fluorescence of all pixels interior to the boundary that is one pixel smaller than the cell boundary
5	<code>a.m1</code>	Area of all pixels interior to the boundary that is one pixel smaller than the cell boundary
6	<code>f.m2</code>	Fluorescence of all pixels interior to the boundary that is two pixels smaller than the cell boundary
7	<code>a.m2</code>	Area of all pixels interior to the boundary that is two pixels smaller than the cell boundary
8	<code>f.m3</code>	Fluorescence of all pixels interior to the boundary that is three pixels smaller than the cell boundary
9	<code>a.m3</code>	Area of all pixels interior to the boundary that is three pixels smaller than the cell boundary
10	<code>f.p1</code>	Fluorescence of all pixels that are one pixel exterior to the cell boundary
11	<code>a.p1</code>	Area of all pixels that are one pixel exterior to the cell boundary
12	<code>f.density</code>	Total fluorescence divided the total area of the cell, minus the background fluorescence ($f.tot/a.tot-f.bg$)
13	<code>ucid</code>	Unique cell id. A variable that identifies cells across different positions; generated as $pos*offset+cellID$, where offset is 100000

- a. Inspect the output images created by Cell-ID and the histograms plotted by the `show.hist` function provided in the package.
- b. Spurious cells are usually odd shaped. The variable `fft.stat` for those cells will be higher than for real, more spherical cells. Using the `QC.filter` function apply a cut over this variable: `fft.stat < [your chosen value]`.

For yeast and mammalian cells (e.g., HEK293 cells) a cut with `fft.stat < 0.3` is usually sufficient to eliminate most spurious cells.

- c. Many times, dead cells exhibit unusually bright fluorescence in one or more channels. Look for these cells and create a cut to limit the maximum fluorescence density (for YFP: $f.tot.y/a.tot-f.bg.y < [your\ chosen\ value]$).
- d. Perform additional cuts in either variable. If the variable distribution is not plotted by `show.hist`, plot it with `chist`, as shown below. Select the cut limits and apply them using the `QC.filter` function.
- e. If the “bf as fl” option was selected when running Cell-ID, use the intensity of the bright-field image in the analysis. Out-of-focus cells usually show a high intensity in the bright-field image, and thus can easily be removed by including in the cut $f.tot/a.tot < [your\ chosen\ value]$ for this channel.

A quality control cut might look like this:

```
#quick overview of some variables distribution.
X<-show.hist(X)
#filter the cells by circularity.
X<-QC.filter(X,fft.stat<0.3)
#cut on fluorescence density of the YFP channel.
X<-QC.filter(X, f.tot.y/a.tot-f.bg.y<100)
#upper and lower cut on cell area.
X<-QC.filter(X, a.tot>100 & a.tot<800)
```

The QC.filter function is cumulative: each time one applies a QC.filter function, it adds to the previous QC.filter; it does not replaced previous QC.filters. To undo one filtering step you can use the undo.QC function (typing X<-undo.QC(X)). If you want to reset all the filters use the reset.QC function (typing X<-reset.QC(X)).

Select cells

The second filtering step is the selection of a subset of cells to continue with the analysis. It differs from quality control in that information from the whole time course can be used to select cells. For example, cells can be selected with fluorescence intensity above a given threshold in every frame, in a specific frame, or in more than a given percentage of the total frames. It is possible to determine union, intersection, and difference of these sets.

27. In time course experiments, select cells with `n.tot` set to all with `select.cells`, and set them as the cells of interest with the `set.cells` function. In this way, you restrict the analysis to cells that are present in all time points.

Doing this will eliminate a lot of the spurious cells, as well as cells that for one reason or another floated in or away from the field, or moved so much during the experiment that Cell-ID assigned it a different ID halfway through the time course. Be aware that the total number of frames in which a cell appears is calculated after the quality control filter is applied. For this reason do not use QC.filter to filter over n.tot.

Unlike QC.filter, set.cells replaces previous set.cells calls. To reset a cell selection, use reset.cells (typing X<-reset.cells(X)).

28. Verify that quality control and cell selection were adequate using the `show.img` function.

It displays the bright-field image generated by Cell-ID for a given position and time frame with the cells that remained in the dataset marked with a cross (Fig. 14.18.6, left).

```
#select the cells that appear in every frame.
n.tot.cells<-select.cells(X,n.tot="all")
#set the selected cells, as the cells to analyze.
X<-set.cells(X,n.tot.cells)
#show a image of Position 1 with the selected
#cells marked.
show.img(X,pos=1)
```

Plot data

Once the data is loaded in R and filtered, the data can be plotted. R has extensive plotting capabilities, and there are several ways in which you can use them to obtain a particular graph. The easiest way is to use the Rcell package plotting functions, explained below.

29. Plot the YFP total fluorescence versus the time frame for the first position, typing `cplot(X, f.tot.y~t.frame, pos=1)` in the R console.

The cplot package function is a wrapper over the R graphic function plot. It filters the argument formula (f.tot.y~t.frame) with the cuts defined by QC.filter and set.cells, and passes it to plot. The package contains several such wrapper

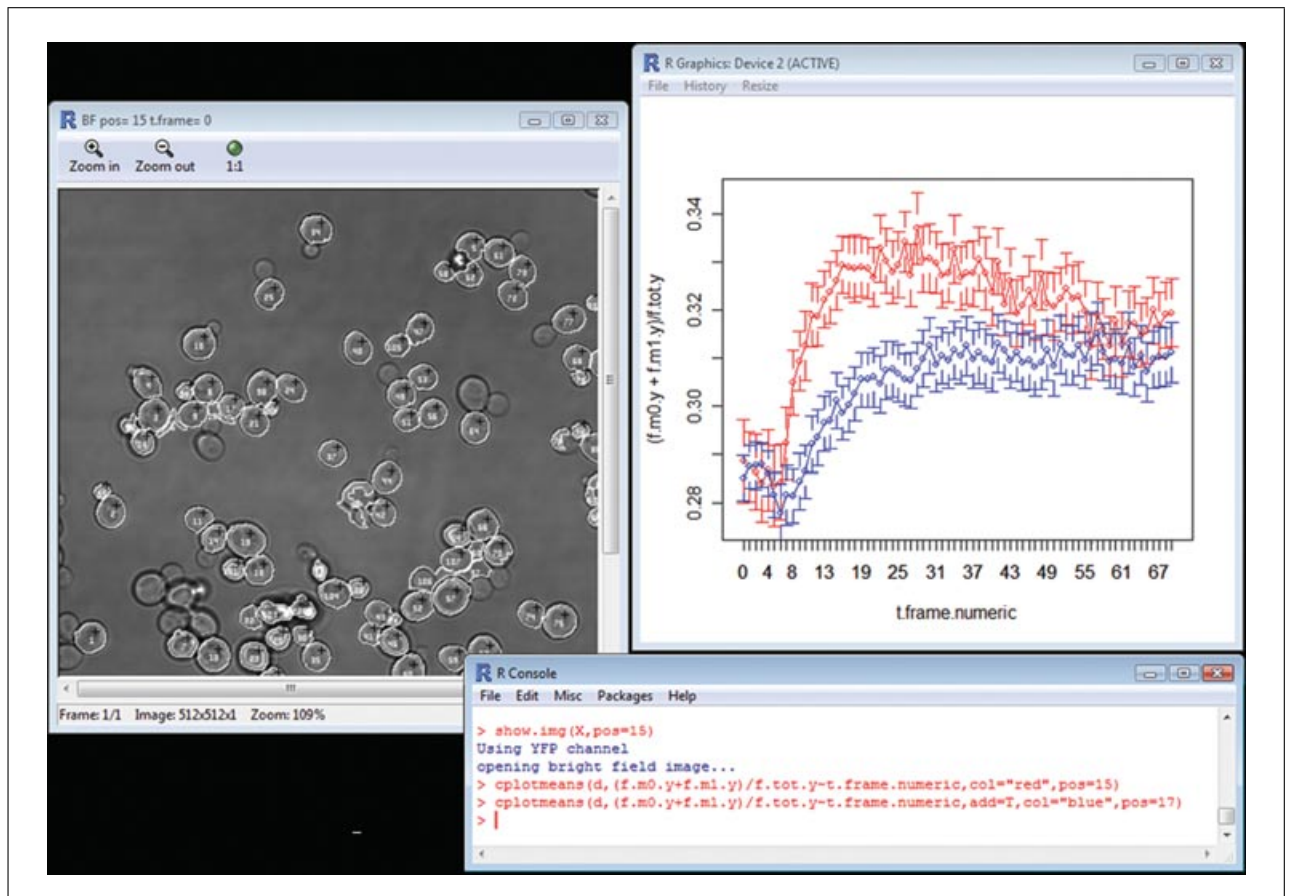


Figure 14.18.6 Plotting with R. The upper left window contains an output of the `show.img` function. Note the crosses on top of the cells that passed the `QC.filter`. The upper right window shows a plot of the output of a composition of two calls to `cplotmeans`. The bottom window shows the R console, with executed commands. For the color version of this figure go to <http://www.currentprotocols.com>.

functions (e.g., `chist` and `cplotmeans`) that operate over `hist` and `plotmeans`, respectively. Note that the postfix of `f.tot` will vary with the channel.

30. Add additional filters using the “filter” argument next to the variables to plot.

This argument is available for all the plotting functions of the package.

```
#plots the total fluorescence over time for
#position 1.
cplot(X,f.tot.y~t.frame,pos=1)
#plots cells bigger than 400 pixels only.
cplot(X,f.tot.y~t.farme,pos=1,filter=(a.tot>400))
# to overlay the plot for small cells over the previous
#plot, in blue:
cpoints(X,f.tot.y ~ t.frame,pos=1,filter=(a.tot<400),
col="blue");
```

“Filter” accepts boolean expressions:

```
#"==" ,"&" ,"|" ,"!" correspond to "equal", "AND",
#"OR" and "NOT", respectively.
cplot(X,f.tot.y ~t.farme,pos=1,filter=(a.tot>400&major.
axis<30))
```

Note that the applied filter will only be applied to the current plot, and will not be saved. You can also select a subset of cells to be plotted with the “cells” argument, followed by

the vector of selected cells, as returned by `select.cells`. This selection will affect only the current plot.

31. To overlay plots use `cpoints` or `clines` after the first call to `cplot`.
32. If desired, export filtered variables of your choice to a tab-delimited file, for further analysis with other programs using `write.table`.

The table to be exported can be created with the `cbind` (column bind) function, which outputs as columns the argument vectors. Use the `fv` (filter vector) function over the created table to apply the cuts defined by `QC.filter` and `set.cells` before exporting with `write.table`. To export the mean value and confidence interval used by `cplotmeans`, export the output of `cprintmeans`.

```
#export a table with t.frame and f.tot.y for the
#filtered cells of position 1 to a file.
write.table(fv(X,cbind(t.frame,f.tot.y),pos=1),
file="myOutput.dat", sep="\t")
#export a table with the mean data used by cplotmeans.
write.table(cprintmeans(X,f.tot.y~t.frame,pos=1),
file="myOutput.dat", sep="\t")
```

33. If desired, create new variables and append them to the dataset, using `append.var` and `append.pos.var`.

Filtering and plotting can be done over the new variables just as over the default ones. The new variable can be a frequently used combination of other variables, thus reducing the amount of code required to produce the desired output. It can also be used, e.g., to group positions together based on some criteria. For example, if you have several positions for the same treatment, you might want to create a “treatment” variable to group the positions together.

```
#creates the nucl.fraction variable.
X<-append.var(X,f.nucl.y/f.tot.y,"nucl.fraction")
#creates a treatment vector which groups 3
#consecutive positions together.
treat<-trunc((X$pos-1)/3)+1
#appends (adds to the dataset) the new
#"treatment" variable.
X<-append.pos.var(X,treat,"treatment")
```

34. Plot a histogram with the distribution of the nuclear fluorescence entering `chist(X,f.nucl.y)` in the console.

You could use the distribution information of this or any other variable to apply new cuts on your data with `QC.filter` or `select.cells`, as shown above.

Calculate plasma membrane-associated fluorescence

35. To calculate the amount of fluorescence associated with the plasma membrane, the R package calculates, based on variables provided by Cell-ID, the values of fluorescence of each color associated with the sum of the pixels in the cell border (`f.m0`), one pixel outside the border (`f.p1`), one pixel towards the interior (`f.m1`), two pixels towards the interior (`f.m2`), and sum of all remaining pixels (`f.m3`). It also provides the area (in pixels) associated with each of these fluorescences. (Note that the variable names are changed in a channel specific manner, so for YFP the above mentioned variables become `f.m0.y`, `f.p1.y`, `f.m1.y`, `f.m2.y`, and `f.m3.y`).

All measures of plasma membrane-associated signal should be compared with an appropriate control using cells in which all fluorescence is cytoplasmic.

Calculations representing membrane-associated fluorescence depend on the experiment. One option is shown in Figure 14.18.6. Other examples include:

```
a. f.m0 - a.m0*f.bg;
b. (f.m0 -- a.m0*f.bg) / (f.tot - a.tot*f.bg).
#plots "a vs time point" for every cell in
#position 3.
cplot (X, (f.m0.y-a.m0*f.bg.y~t.frame), pos=3)
#plots "the mean of a vs time point" in position 3.
cplotmeans (X, (f.m0.y-a.m0*f.bg.y~t.frame), pos=3)
#plots "b vs time point" for every cell in
#position 3.
cplot(X, ((f.m0.y - a.m0*f.bg.y) / (f.tot.y - a.tot *
f.bg.y)) ~ t.frame), pos=3)
```

Expression **a** is linearly proportional to the membrane-associated fluorescence, but sensitive to photobleaching. Expression **b** is a ratio of fluorescence values and therefore independent of the total fluorescence level. This makes the expression relatively insensitive to photobleaching, but it is not linear with the membrane fraction of fluorescence over the entire range of fraction of fluorescence associated with the membrane.

Calculate nuclear fraction of fluorescence

36. To calculate the nuclear fraction for a given channel, determine the ratio of nuclear to total fluorescence ($(f.nucl - a.nucl * [auto]) / (f.tot - [auto] * a.tot)$). Cell-ID calculates both the total fluorescence associated with a cell ($f.tot$), and the nuclear fluorescence ($f.nucl$) for each channel (e.g, for YFP, $f.tot.y$ and $f.nucl.y$).

The parameter “auto” is not a variable directly available to the user. It is the average intensity ($f.tot/a.tot - f.bg$) observed in cells that do not express fluorescent proteins in that channel, and it should be determined experimentally in each case. To do this, include in your experiment a position with cells devoid of fluorescent proteins. Remember to add the channel specific postfix to the variable name.

MEASURING FRET IN SINGLE CELLS USING A BEAM SPLITTER

This protocol describes how to employ Cell-ID to quantify intensity-based fluorescence resonance energy transfer (FRET), using Cell-ID’s capabilities to extract data from split images containing simultaneous emission from donor and acceptor fluorophores during donor fluorophore excitation. This method has advantages over sequential image collection of both donor and acceptor emission after donor excitation: reduced error in donor and acceptor emission by elimination of variation in donor excitation during separate exposures (both in time period and lamp strength during the exposure), reduced number of operations and increased speed, reduced donor fluorophore photobleaching, and reduced phototoxicity.

The protocol below refers to FRET measurements that are made between CFP donor and YFP acceptor fluorophores. The actual colors depend on optimizing spectral characteristics of the excitation and emission filters and the dichroic mirror.

We have successfully used this protocol to measure changes in FRET in the nucleus and in the plasma membrane of yeast (Yu et al., 2008). To locate the nucleus, one can label the nucleus with a separate color such as RFP. Alternatively, if one of the FRET partners is localized to the nucleus, it is possible to use, for example, the YFP excitation/YFP emission image to locate the nuclei and simultaneously quantify the amount of acceptor fluorophore. In the case of the cell membrane FRET, Cell-ID locates the membrane pixels using the bright-field image.

ALTERNATE PROTOCOL

In Situ
Hybridization
and Immuno-
histochemistry

14.18.17

We have not measured FRET in particular locations using a separate color (e.g., nuclearly localized RFP to identify the nucleus) with Cell-ID and an image splitter configured as described below. To measure FRET in a location that is marked by a color distinct from the donor or acceptor fluorophores, one should perform sequential image processing with four different images: donor excitation/donor emission; donor excitation/acceptor emission (FRET); acceptor excitation/acceptor emission; and location label color (different from donor or acceptor excitation and emission wavelengths).

Additional Materials (also see Basic Protocol)

Hardware

Image splitter with an appropriate dichroic mirror and top and bottom emission filters, e.g., for CFP/YFP FRET, a Dual View image splitter (Optical Insights/Photometrics, <http://www.photometrics.de>) fitted with the following filters and dichroic mirrors (Chroma): CFP emission, 480/30M; YFP emission, 535/20M; dichroic mirror, DX505.

Fluorescence microscope: same as in Basic Protocol, except remove the emission filter from the cube used to excite the donor fluorophore (e.g., for CFP/YFP FRET, remove the emission filter from the CFP cube); referred to as the FRET cube

Set up image splitter

1. Attach the image splitter to the microscope, and attach the camera to the image splitter.
2. Cell-ID expects the image to be horizontally split so that the sub-images are on the top and bottom. Orient the camera relative to the image splitter so that the top sub-image is CFP emission and bottom sub-image is YFP emission image (recommended).

This orientation will be assumed for the rest of this protocol.

3. Test the image orientation by taking test bright-field images with the YFP cube selected. *Ensure that the top half of the image is dark and the bottom half is not.* Take another test bright-field image with the FRET (modified CFP) cube.

Now both the top and bottom half should have an image.

4. On the image splitter, adjust the positions of the two halves of the image so that a thin horizontal black region separates the two sub-images halfway between the top and bottom.

Prepare samples

5. Follow steps 1a to 9a in Support Protocol 2 for preparing cell samples.

Acquire images

6. Remove the rapidly photobleachable autofluorescence by performing a number of prebleaching image sets. Establish the number by comparing the photobleaching curve of the autofluorescence (using the parental yeast strain not expressing any fluorescent protein) with that of the strain to be used in the FRET experiment.

This procedure can be useful in any number of other applications.

Yeast contain various fluorescent compounds, which together constitute cellular autofluorescence; this reduces the ability to distinguish the real signal. Typically, there are two groups of autofluorescent compounds, those that photobleach faster than YFP or CFP and those that are essentially photobleaching-resistant. When the signal to be measured is small, as in a FRET experiment, it is a good idea to reduce autofluorescence as much as possible.

7. Follow steps 1 to 5 in the Basic Protocol for determining exposure settings. Keep in mind that each time point for each image field requires two exposures, a FRET exposure and an acceptor fluorophore exposure.

8. Acquire a bright-field image, following step 6 in the Basic Protocol.
 - a. Use the FRET cube to ensure that the cells appear in both the top and bottom image panels.
 - b. Label the image `BF_Position[X].tif`, where [X] is a number. Make sure all of numbers [X] have the same number of digits, e.g., for between 1 and 99 images, label them 01, 02, 03, etc.; for between 1 and 999 images, label them 001, 002, 003, etc.
 - c. If taking multiple time points per position, name the file `BF_Position[X]_time[Y].tif`; in this case, also make sure that the numbers [Y] have the same number of digits.
9. Acquire an acceptor excitation image using the YFP cube. Name it `YFP_Position[X].tif` or `YFP_Position[X]_time[Y].tif`, if taking multiple time points. The fluorescence will only show on the bottom half of the image.
10. Acquire a donor excitation image using the FRET cube. Name it `CFP_Position[X].tif` or `CFP_Position[X]_time[Y].tif`; again, ensure that all [X] and [Y] values have the same number of digits.
11. For each FRET measurement desired, repeat steps 7 to 9 for each time point and image field.
12. After collecting all images for all positions and all time points, place all images into the same folder.

Process images with Cell-ID

13. Proceed to load images as described in steps 12 to 14 in the Basic Protocol. For example, for a typical nuclear CFP/YFP FRET experiment set the following parameter values given filenames as described above:

Position token: `Position`
 Time token: `time`
 sep. character: `_`
 brightfield: `BF`
 Fluorescent: `FP`
 nuclear image: `YFP`.

14. Set other options as described in steps 14 and 15 in the Basic Protocol.

NOTE: The “uneven illumination image” is generally ignored. The typical FRET metrics are ratiometric in nature (i.e., they take some ratio of CFP emission and YFP emission from the top and bottom halves of the FRET image, respectively), which reduces or eliminates effects from uneven illumination as a function of position in the image typically.

15. Set up segmentation according to step 16 in the Basic Protocol, except that the background reject factor is better if set lower for identifying cells in split brightfield images. Start at values from 0.5 to 0.8.

Spuriously identified cells can be filtered during later analysis.

16. Test to see if Cell-ID is properly identifying cells in the split brightfield image, as outlined in steps 17 to 20 in the Basic Protocol. Cell-ID by default tries to match pairs of equivalent cells in the top and bottom images. If only one partner is found in one of the image halves, then Cell-ID creates the partner in the other image half by copying the outline of the found partner to the top. Cell-ID calculates a predicted position and offset (i.e., translation and rotation from the found pair)

based on how matched top and bottom pairs relate to each other in the rest of the image.

17. Start batch calculation as described in step 22 in the Basic Protocol.

Analyze data with R

When performing split image FRET data extraction, Cell-ID outputs essentially the same variables as described in Table 14.18.1 in the Basic Protocol, except that in addition to a color identifier there is also a sub-image identifier. When the output files are loaded in R, the variables are renamed taking into account the channel and sub-image type (except for `pos`, `t.frame`, and `cellID`). A two letter postfix is appended to the fluorescent related variable name, the first one indicating the fluorescence channel as explained above, and the second one indicating if it is a upper sub-image (u) or a lower sub-image (l) cell. For nonfluorescent related variables, only the sub-image identifier is appended. For example, for an experiment with brightfield, CFP, and YFP images, the `f.tot` is converted into four variables: `f.tot.cu`, `f.tot.cl`, `f.tot.yu`, and `f.tot.yl`. Some variables, such as `a.tot` are typically the same for a given cell in both sub-images. In this case, for analysis, simply use one (i.e., `a.tot.u`).

18. Proceed with data analysis as outlined in steps 23 to 31 in the Basic Protocol to examine basic cell properties (total fluorescence, area, etc.).

SUPPORT PROTOCOL 1

OBTAINING AND INSTALLING CELL-ID AND R

Cell-ID, Vcell-ID and R are free, open-source software that can be readily downloaded and installed in several platforms (Windows, Mac-OS, and Linux). Here we outline how to install these programs and required packages.

Materials

Hardware

PC workstation with UNIX, LINUX, or Windows XP or higher operating systems;
or Apple workstation with Mac OS X operating systems and X11 installed

Install R and Rcell

1. Download and install the latest binary distribution of R appropriate for the operating system being used, from the download section of the R-project Web page (<http://www.r-project.org>).
2. Download and install the latest binary release of ImageMagic for the operating system being used from <http://www.imagemagick.org>.
3. Download and install GTK+ from <http://www.gtk.org/download.html>.
4. Download the latest version of the Rcell package from <http://lbms.df.uba.ar> and save it. Take note of the file path.
5. Open R Console. To install “gplots” and “lattice” from external CRAN repositories, type the following:

```
install.packages("gplots")  
install.packages("lattice")
```

Alternatively, install them using the Packages->Install Packages menu of the R GUI. These packages provide additional functionality, but are not required by Rcell.

6. To install EBImage, type at the console:

```
source("http://bioconductor.org/biocLite.R")
biocLite("EBImage")
```

If there is a problem during the installation of the EBImage package, download it from <http://www.bioconductor.org/packages/release/Software.html>, where there is an installation guide and a description of the package.

7. Then install the Rcell package, typing at the console:

```
install.packages("C:/.../Rcell_0.1-x.zip",
  repos=NULL)
or select "Install package from local zip file ..." from the Packages menu of the
R console and select Rcell_0.1-x.zip
or type the following at the prompt of a new X11 terminal type:
R CMD INSTALL Rcell_0.1-x.tar.gz
```

Install Cell-ID and VCell

8. Download source code for Cell-ID and VCell from the Cell-ID Web site at <http://lbms.df.uba.ar>, along with required dependences and installation guides.

An installation wizard for windows is also available.

PREPARING YEAST AND MAMMALIAN CELLS FOR IMAGING

The exact culture conditions depend on the experiment. Below are standard procedures for obtaining an exponentially growing yeast cell population or mammalian cell population that will display low autofluorescence. This protocol works for many mammalian cell types, including HEK293, 3T3, and HeLa.

Materials

Yeast cultures

Appropriate growth medium for required yeast auxotrophies

Synthetic medium (also know as complete minimal medium; see *UNIT 13.1*)

supplemented with all amino acids (except for those omissions required to select for plasmids, if any) and 20 µg/ml of adenine

1 mg/ml concanavalin A (Sigma) in phosphate-buffered saline (PBS; *APPENDIX 2*)

CaCl₂

MgCl₂

Yeast cell culture medium containing an appropriate stimulus

2% or 4% (w/v) paraformaldehyde in PBS (*APPENDIX 2*) pH 7, 4°C

LB medium (*UNIT 1.1*) or YPD medium (*UNIT 13.1*)

PBS (*APPENDIX 2*)

1 mg/ml poly-L-lysine solution (Sigma)

Mammalian cell cultures (e.g., HEK293, 3T3, HeLa)

Mammalian cell culture medium containing an appropriate stimulus

50-ml glass tubes, sterile

30°C incubator

Rotating wheel *or* shaker for test tubes

Imaging plate appropriate for microscope

96- or 384-well glass-bottom plates

96-well plastic cell culture plates

Temperature and gas-controlled incubator mounted on the microscope (see Basic Protocol) for imaging live mammalian cells

Additional reagents and equipment for trypsinizing adherent cells (e.g., see *APPENDIX 3F*)

Prepare yeast cells

- 1a. Two days before the experiment, grow yeast overnight at 30°C in the appropriate medium for the required auxotrophies.
- 2a. In the morning, dilute the cells to 0.05 OD₆₀₀ in fresh medium. Incubate at least 6 hr at 30°C.

The goal is to shift the culture to exponential growth.

- 3a. During the incubation time, prepare four 50-ml glass tubes with 5 ml synthetic medium supplemented with appropriate amino acids and 20 µg/ml of adenine.

Adenine will repress the adenine biosynthesis pathway, which in many strains results in fluorescent by products that accumulate in the vacuole.

Avoid YPD or other rich media, since they are usually highly fluorescent.

- 4a. Measure the OD₆₀₀ in the afternoon and dilute the sample to OD₆₀₀ = 1×10^{-3} in the first tube. Perform 1:10 serial dilutions in the three remaining tubes. Incubate the tubes overnight at 30°C on a rotating wheel or shaker.

The goal is to have one of the tubes at OD₆₀₀ 0.1 the next morning.

Assuming a normal doubling time of 90 min, 15 hr of shaking will increase the OD 2¹⁰ (~1000) times. In this case, the 2nd tube, with a starting OD₆₀₀ = 1×10^{-4} will be used later.

Image live yeast cells

Use the steps immediately below to image live cells. To image fixed cells go to step 12a.

- 5a. The morning of the experiment, or the day before, add 100 µl of 1 mg/ml concanavalin A solution to multiwell glass-bottom plates. Incubate at least 30 min or up to overnight at room temperature.
- 6a. Wash the plates three times with water.

These plates are good for at least 1 day if kept wet.

- 7a. Add 200 µM Ca²⁺ and 100 µM Mn²⁺ to the cells in the tubes.

This will improve the interaction of concanavalin A with the cell wall sugars.

- 8a. Add 100 µl of yeast culture of OD₆₀₀ 0.1 to the wells. Wait 10 min and wash away unbound cells three times with fresh medium.

- 9a. Secure the plate tightly in the microscope stage.

This is very important if you want to stimulate or otherwise add or remove medium from the wells DURING an experiment.

The cells should be ready for live microscopy.

- 10a. To stimulate cells, pipet out the medium by carefully placing the tip on one of the corners of the well, then quickly replace with medium containing the appropriate stimulus.

- 11a. Process and analyze the cell images as in the Basic Protocol or the Alternate Protocol.

Image fixed yeast cells

- 12a. Perform the experiment as usual, and take samples at the desired time points. For every 1 ml of sample, add 1 ml of ice cold 4% paraformaldehyde and incubate for 1 hour on ice.

13a. Add 50 μ l of sterile LB (or YPD) to each sample.

Yeast do not centrifuge well if the sample is diluted and the experiment was done using synthetic medium. Adding LB largely corrects this problem.

14a. Centrifuge 20 sec at top speed in a microcentrifuge. Discard the supernatant and add 500 μ l PBS.

15a. Repeat step 14a two more times.

16a. Load the cells (100 μ l or 20 μ l if using a 96- or a 384-well plate, respectively) onto the imaging plate and process and analyze the cell images as in the Basic Protocol or the Alternate Protocol.

Prepare mammalian cells

1b. Add 100 μ l of 1 mg/ml poly-L-lysine solution to each of the wells of a 96-well plate and incubate for at least 30 min at room temperature.

2b. Wash the plates with PBS and store up to several days at 4°C.

3b. Split the cell culture one or two days before the experiment, so that the cells are actively growing.

Use cells that are not confluent, so that there is enough space between cells for Cell-ID to locate individual cells and to calculate the background fluorescence. Live imaging is limited to cells that can be located by Cell-ID in a brightfield image. It works well with cells that do not spread on surfaces (e.g., lymphocytes) but not with cells with extensive spreading and processes (e.g., epithelial or neuronal cells). Images of cells that spread on a two-dimensional surface do not produce enough contrast for cell border identification by Cell-ID. In future implementations, Cell-ID will be able to find the borders of cells with fluorescently labeled membranes.

Tissue culture medium is usually fluorescent, thus reducing the ability to image low-level signals. Try different media (e.g., medium without phenol red) or use medium diluted to half with PBS.

Image live mammalian cells

Use the steps immediately below to image live cells. Proceed to step 7b to image fixed cells.

4b. Set up the plate with the cells on the microscope stage, making sure that it is tightly secured.

This is very important if you want to stimulate or otherwise add or remove medium from the wells DURING an experiment.

Cells are ready for imaging.

5b. To stimulate cells, pipet out the medium by carefully placing the tip on one of the corners of the well, and then quickly replace it with medium containing the appropriate stimulus.

6b. Process and analyze the cell images as in the Basic Protocol or the Alternate Protocol.

Image fixed mammalian cells

7b. Perform the experiment as usual, and take samples at the desired time points.

8b. Remove the medium. Trypsinize and collect the cells (e.g., see APPENDIX 3F).

9b. Resuspend the cells in 1 ml ice-cold 2% paraformaldehyde per million cells. Incubate 1 hr on ice.

- 10b. Remove the paraformaldehyde by centrifuging 10 min at 1000 rpm in a benchtop centrifuge.
- 11b. Discard the supernatant and wash the cells two times with 2 ml PBS, centrifuging as in step 10b after each wash.
- 12b. Resuspend the cells in 100 μ l of PBS. Use 1 μ l to count the cells.
NOTE: *You can store these cells at 4°C.*
- 13b. Dilute the cells appropriately in 100 μ l of PBS and load in one poly-lysine-precoated (step 1b) well of a 96-well plate.
NOTE: *Usually, 20,000 to 50,000 cells per well is a good density.*
- 14b. Process and analyze the cell images as in the Basic Protocol or the Alternate Protocol.

**SUPPORT
PROTOCOL 3**

**CALCULATING NUCLEAR AND PLASMA MEMBRANE CFP-YFP FRET
USING SPLIT IMAGES**

There are a number of published formulas for calculating FRET based on intensities measured from microscopic images (Gordon et al., 1998; Xia and Liu, 2001).

Perform FRET calculation

1. For situations in which the CFP and YFP are attached to different proteins with different abundances, use the N_{FRET} metric (Xia and Liu, 2001):

$$N_{\text{FRET}} = \frac{CY}{\sqrt{CC \times YY}}$$

Equation 14.18.1

where the first letter in CY, CC, and YY refers to the excitation wavelength and the second to the emission wavelength used, respectively.

CC, CY, and YY are measured in the region in which one wants to calculate FRET, such as the membrane, nucleus, or for the whole cell (see below for more details).

2. Correct the three measures (CC, CY, and YY) for background and cross-talk, using the following equations:

$$\begin{aligned} CY &= CY_{\text{obs}} - B_{CY} - A_{CC} \rightarrow CY(CC_{\text{obs}} - B_{CC}) \\ CC &= CC_{\text{obs}} - B_{CC} - A_{CY} \rightarrow CC(CY_{\text{obs}} - B_{CY}), A_{CY} \rightarrow CC \sim 0, \\ &\text{thus } CC = CC_{\text{obs}} - B_{CC} \\ YY &= YY_{\text{obs}} - B_{YY} \end{aligned}$$

where CY_{obs} , CC_{obs} and YY_{obs} are the values calculated by Cell-ID for the region of interest;

B_{CC} , B_{CY} , and B_{YY} are the background cellular fluorescence in each emission channel;

$A_{CC} \rightarrow CY$ and $A_{CY} \rightarrow CC$ are the cross-talk coefficients of CFP into the YFP channel and of YFP into the CFP channel (see step 3 for cross-talk coefficient calculations).

For nuclear FRET, a good estimate of B_{CC} , B_{CY} , and B_{YY} is the average fluorescence of non-nuclear pixels, defined by all intracellular pixels outside of a circle with a radius of 6 centered on the calculated center of the nucleus (or by the intracellular pixels 3 pixels away from the border of the cell). In our experience, these are good per cell representations of background signal. Note that the radius of the circle needed for nuclear fluorescence quantification may change between different cell types and microscope setups.

For membrane FRET, the signal in each channel is less affected by background cellular fluorescence; in this case, local backgrounds away from the cell can be used.

The cross-talk coefficients allow calculation of how much signal one should expect to observe, for example, in the YFP channel due to CFP protein emission. The exact magnitude of the cross-talk coefficients $A_{CC \rightarrow CY}$ and $A_{CY \rightarrow CC}$ depends on the particular illumination and filter cubes used.

3. To calculate the cross-talk coefficient of CFP into the YFP channel $A_{CC \rightarrow CY}$, use a solution of purified CFP and take the ratio of YFP emission channel signal to CFP emission channel signal in a split image obtained with the FRET cube.

For the image splitter configured as outlined above, this is the ratio of the bottom image signal to the top image signal. Using recombinant CFP we obtained 0.99, which shows that there is significant emission cross-talk of CFP into the YFP emission channel due to its broad emission spectrum (spectral bleed-through). For example, an image of a sample containing purified CFP protein, using CFP excitation wavelengths, will show up as a measurable signal in the YFP emission channel. Conversely, the emission cross-talk of YFP protein into the CFP emission channel with the optics outlined here is negligible.

Measure nuclear FRET in yeast

4. To measure nuclear FRET, use the following quantities in Equation 14.18.1.

For CC:

$$CC_{\text{obs}} = f.\text{nucl}.\text{tag3}.\text{cu}/a.\text{nucl3}.\text{yu}$$

$$B_{CC} = (f.\text{nucl}.\text{tag7}.\text{cu} - f.\text{nucl}.\text{tag6}.\text{cu}) / (a.\text{nucl7}.\text{yu} - a.\text{nucl6}.\text{yu})$$

For CY:

$$CY_{\text{obs}} = f.\text{nucl}.\text{tag3}.\text{cl}/a.\text{nucl3}.\text{yl}$$

$$B_{CY} = (f.\text{nucl}.\text{tag7}.\text{cl} - f.\text{nucl}.\text{tag6}.\text{cl}) / (a.\text{nucl7}.\text{yl} - a.\text{nucl6}.\text{yl})$$

For YY:

$$YY_{\text{obs}} = f.\text{nucl}.\text{tag3}.\text{yl}/a.\text{nucl3}.\text{yl}$$

$$B_{YY} = (f.\text{nucl}.\text{tag7}.\text{yl} - f.\text{nucl}.\text{tag6}.\text{yl}) / (a.\text{nucl7}.\text{yl} - a.\text{nucl6}.\text{yl})$$

Measure plasma membrane FRET in yeast

5. To measure membrane FRET, use the following quantities in Equation 18.14.1.

For CC:

$$CC_{\text{obs}} = f.\text{m0}.\text{cu}/a.\text{m0}.\text{u}$$

$$B_{CC} = f.\text{local}.\text{bg}.\text{cu}$$

For CY:

$$CY_{\text{obs}} = f.\text{m0}.\text{cl}/a.\text{m0}.\text{l}$$

$$B_{CY} = f.\text{local}.\text{bg}.\text{cl}$$

$$A_{CC \rightarrow CY} = 0.99$$

For YY:

$$YY_{\text{obs}} = f.\text{m0}.\text{yl}/a.\text{m0}.\text{l}$$

$$B_{YY} = f.\text{local}.\text{bg}.\text{yl}$$

COMMENTARY

Background Information

Measurements in single cells can reveal information obscured in population averages. For example, studies of variation in gene expression in single *Escherichia coli* and *Saccharomyces cerevisiae* (Elowitz et al., 2002; Raser and O'Shea, 2004) have shown that only a fraction of cell-to-cell variation in the expression of reporter genes results from stochastic fluctuations in the workings of the gene expression machinery (Colman-Lerner et al., 2005; Pedraza and van Oudenaarden, 2005; Rosenfeld et al., 2005), and they have identified other processes and genes that account for and control the bulk of the variation (Colman-Lerner et al., 2005).

One means for collecting single cell data is flow cytometry. The instrument interrogates cells as they pass individually through an apparatus that excites fluorophores with a laser and collects the emitted light. These instruments are powerful (Shapiro, 2003; Bonetta, 2005) because they can automatically measure many hundreds or thousands of cells per second. However, flow cytometry has disadvantages. These methods (1) cannot interrogate individual cells repeatedly to produce time series for each cell; (2) cannot collect a great deal of light, due both to the short time (typically μsec) that the cell passes the detector, and to the numerical aperture of the objective, which often collects less than 10% of the emitted light; and (3) typically do not capture images of the cells, making it difficult or impossible to analyze cell shape, size, and intracellular localization of fluorescence.

Optical microscopy complements some limitations of flow cytometry. Optical microscopy provides the ability to revisit individual cells over time, excite cells, and collect emitted light for long times, and capture cell images with high resolution. Automation by computer-aided cell tracking and image analysis enables generation of such data with high throughput.

Recent research-directed, automated, microscope-based cytometry outside of clinical and pharmaceutical applications has relied on commercial software packages, e.g., Metamorph (Molecular Devices; see Inoue and Inoue, 1986; Schnapp, 1986; Gonzales and Woods, 2002) and ImagePro (Media Cybernetics), to operate the microscopes, collect the images, and analyze images. These packages, often used together with general purpose analysis programs, such as Matlab

(Gonzales et al., 2003) and Labview (Bishop and Inc, 2005) probably constitute the state of the art in commercial software used for these purposes. The ability to perform microscope-based cytometry has recently been aided by open-source projects, in particular the Open Microscopy Environment (OME), which provides file formats and metadata standards for microscope images (Swedlow et al., 2003; Goldberg et al., 2005); Image J, a Java-based package of microscope image analysis tools (Abramoff et al., 2004); and CellProfiler (UNIT 14.17).

This unit describes a suite of user-modifiable technical and analytical methods to facilitate accurate, high-throughput measurements from single cells over time. Although Cell-ID was originally tuned to work with yeast cells, it has readily identified live mammalian lymphoid cells and trypsinized, live, HEK293 cells. When used with inexpensive optical microscopes and high-quality CCD equipment, these methods enable fluorescence measurements slightly more sensitive than those from contemporary flow cytometers (e.g., BD LSRII) and have allowed scoring, quantification, and extraction of meaningful statistics from one or two fluorescent proteins molecules per pixel in single cell images (Gordon et al., 2007).

Because it is open source, Cell-ID can be extended to work with any cell type that has an optically identifiable cell boundary. Cell-ID should also be applicable to different image types, e.g., phase-contrast, differential interference contrast, or even fluorescence images, although for the last case our work suggests that a preprocessing step might be necessary to determine the boundaries of the fluorescent regions.

Critical Parameters

Acquisition

z control and the brightfield image. It is critical to find consistently the dark ring in a defocused image. For example, image-to-image differences in z position in a time course will result in differences in the area assigned to the same cell over time, even if the cell really did not change its volume.

Camera specifications. Dynamic range is critical for imaging cells with large differences in FP levels. Sensitivity is important for reducing photobleaching when measuring time courses.

Objective. High numerical aperture is essential in order to image low levels of light.

Exposure time. This is critical to balance photobleaching and phototoxicity against higher signal-to-noise ratio.

Cell-ID

The value for the “background reject factor” parameter might make a difference between Cell-ID finding or missing cells in the bright-field image, as can be seen in Figure 14.18.5.

Troubleshooting

If cells are not identified, try defocusing the bright-field image further to increase the width of the dark ring around the cell border. Also try reducing the background reject factor.

Anticipated Results

Cell ID should have a very high rate of picking new cells and tracking them through multiple images from a time course.

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