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Cell-ID Software for Microscope-Based Cytometry

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Abstract

This unit describes a method to quantify, from sets of microscope images, various cellular parameters from individual cells, and includes procedures to track cells over time. For example, the user can measure cell volume, total and subcellular localization (nuclear, plasma membrane) of fluorescence for multiple fluorescence channels. 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 (sometimes referred to as brightfield, or BF) that will be used to locate each cell, and one fluorescence image for each of the color channels to be analyzed. Images may be conventional wide-field epifluorescence or confocal microscopy images. Cell-ID processes the images and outputs 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 we have supplemented with a package tailored to analyze Cell-ID output.

Keywords

image processing; fluorescence microscopy; Cell-ID; R

Quantification of molecules and reaction rates in single cells using microscope-based cytometry with Cell-ID and R

This unit describes a method to quantify, from sets of microscope images, various cellular parameters from individual cells, including procedures to track cells over time. Using this method (Colman-Lerner et al., 2005; Yu et al., 2008), the user can measure, for example, total and subcellular localization (nuclear, plasma membrane) of fluorescence for multiple fluorescence color channels. 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 (sometimes referred to as brightfield, or BF) that will be used to locate each cell, and one fluorescence image for each of the color channels to be analyzed (xFP, where x designates the color). Images may be conventional wide-field epifluorescence or confocal microscopy images. 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, and 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 non-adhering 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 to load these files to R, filter out unwanted cells, display images, and plot simple and compound variables.

This unit will focus on procedures to measure fluorescence protein-based reporters. However, nearly identical approaches can be used when measuring signals from fluorescent antibodies or light emitting enzymes.

Basic Protocol

Extracting quantitative information from single cells

In this protocol, Cell-ID and R are employed to process and analyze time course experiments, where the same cells in 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 in the different time points are not the same, such as when one takes different samples from a culture at different times, then 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 in 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, such as 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, 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 to measure small signals close to the levels of noise, which are best detected as changes over time.

To measure fluorescence energy transfer (FRET) using split images, see Alternate protocol.

Materials

Hardware—Fluorescence microscope, preferably inverted to allow access to live cells during imaging over time

- Motorized Z control. Experiments can be done with manual focus, but become extremely tedious and are less reproducible.
- Motorized shutter for fluorescence illumination. This is very important to be able to control exposure times precisely. Optionally, motorized shutter for transillumination (brightfield illumination).
- Objective: high numerical aperture (NA, e.g., >1.2 for 60× objective) to capture as much light as possible, and chromatically corrected (at least PlanApo objectives), which is especially important for work that requires subcellular colocalization
- Black-and-white, cooled CCD camera (when using non-confocal microscope). Color cameras should not be used for quantitative imaging.
- Appropriate filter cubes or wheels.
- Optional: Motorized *xy* stage (for live imaging of multiple fields of view, for example, in neighboring wells with cells treated differently)
- Optional: Motorized filter cubes/wheels and motorized *z* position. In combination with motorized stage, this option allows complete automation of imaging.
- PC to run microscope.
- Acquisition software: depending on hardware, the user might need commercial packages such as Metamorph or ImagePro, or might take advantage of the open-source microscopy software initiative at UCSF, *µmanager* (<http://www.micro-manager.org>).

Optically appropriate support for cells: Multiwell plates are preferred over slides and cover slips, since they allow access to samples during the experiment.

- For oil immersion with high-NA objectives, use glass-bottom, cover-slip thin plates. 96- and 384-well plates can be obtained from Arctic White (Bethlehem, PA).
- For air or water immersion objectives, plastic multiwell plates work well.

Unix workstation, PC with Windows XP or higher, Linux, or Apple with MacOSX with X11 installed, to run Cell-ID and R.

Software—Cell-ID (Gordon et al., 2007), R (R-Development-Team, 2008) and the package Rcell. See Support Protocol 1 for download information.

Image files—The images supplied to Cell-ID should be in TIFF format. Cell-ID reads gray-level TIFF files of 8 and 16 bits. If the image file was generated by Metamorph, time-related information can be extracted from the image (see below).

Acquire images

- 1 First 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.

Determine exposure settings for experiment

- 2 Prepare cells that 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-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 best focal plane 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 fluorescence 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, such as ImageJ and Metamorph, moving the cursor over image will display the x-y coordinates and the intensity of the pixel. Values for saturated pixels depend on the image depth: 256 for 8 bit, and 65536 for 16 bit images.
- 6 The times determined in 4 and 5 are the minimum and maximum exposure times. In some cases, the exposure time determined in 4 is higher than that of 5. In this case, you need a CCD camera with larger dynamic range, or you will have to change exposure time during the experiment (although possible, this option will complicate analysis).

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

Steps 7 and 8 represent images from one set of images, for one position and one timepoint.

- 7 Acquire the brightfield image.
  - a. Take an image with the cells at the focal plane using transmitted light.
  - b. Move the Z knob so that the stage moves slightly down (towards the objective) until you notice that a dark ring surrounding the cells (Figure 14.18.1).
  - c. Record how much you had to move the stage. For a 60× objective this is usually 100–200 nm.
  - d. Take an image again. This is the brightfield 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; for example, if you have between 1 and 99 images, label them 01, 02, 03...; if you have between 1 and 999 images, label them 001,002,003... etc. If you follow

the SAME cells over time, and will take 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.

Considerations for confocal microscopy—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 sub-cellular compartments. In addition if your 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. To be able to find the cells with Cell-ID a brightfield 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 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 (for example, using a Kalman filter), or as preprocessing step before executing Cell-ID.

- 8 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 you need. Use the settings determined in steps 4–6, 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 7. For example, RFP_Position01_time001.tif, YFP_Position01_time001.tif and CFP_Position01_time001.tif.
- 9 Repeat steps 7 and 8 for as many positions and time points that you have. Number each image set consecutively.
(10 and 11 are optional)
- 10 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 4–6. Label these images appropriately; for example, YFP-dark.tif, CFP-dark.tif and RFP-dark.tif for YFP, CFP, and RFP image exposure times, respectively.
- 11 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, for example, YFP-flat.tif, CFP-flat.tif and RFP-flat.tif.

Usually rich culture medium, such as 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 media is significantly autofluorescent, you should generally take care to avoid X-Y positions that are near the edges of the wells where there is a meniscus.

- 12 Place all images in the same folder.

Process images with Cell-ID

- 13 Start a Cell-ID session. PC users can click on the VCell-ID icon. For Linux and Macintosh users, start Cell-ID from an X11 terminal typing at the prompt: "vcellid &".

The VCell-ID window will appear. The main Cell-ID window has several tab buttons across the menu.

- 14 Select "Setup→Load Images". The "Load Images" window will appear (Figure 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.
  - b. In the drop down menu next to "group images by", select how Cell-ID should map brightfield images to fluorescence images. Select "Metamorph" if you used Metamorph to acquire the images. Otherwise, select "file name pattern".

With the "Metamorph" option Cell-ID will use the brightfield 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 brightfield 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. If you have not named the images as suggested in 7 and 8, then change the *position* and *time* token, and the *separator character* to match those of your images. If there are not time points in your experiment, uncheck "time token".
  - d. If you have dark and flat images, include the token. In the examples of 10 and 11, these would be *dark* and *flat*.
  - e. Click OK to load the images and to close this window.

- 15 In the main window you can now explore the images in your experiment by using the experiment tree shown on the left panel. The experiment is now organized based on Positions. Clicking on an image file, will load it on the central window. Now you can zoom in/out.

- 16 Select "Setup→Image Setup". The "Image Setup" window will appear (Figure 14.18.3).
  - a. Check "bf as fl" if you want the brightfield images to be treated as fluorescence images. *This is sometimes useful to filter out out-of-focus cells, since these cells appear brighter in the brightfield image.*
  - b. If you labeled the cell nucleus with a fluorescent color and you want Cell-ID to find it, check "nucleus from channel" and type the image token corresponding to it, for example "YFP".

- c. If you are performing a split image FRET experiment, check “split fret image”. Proceed to Alternate Protocol.
- d. Select “PAW friendly output” if want to analyze the data with PAW (www.cern.ch/paw).
- e. You can tell Cell-ID what string to add to the output TIFF images, the default is “out”.

17 Select one brightfield image on the tree and right-click (option-click in Mac’s X11). Select “Show Test Dialog” on the popup menu. The segmentation window will appear. *The choice of values in this window will profoundly affect how Cell-ID finds cells.*

- a. “max dist over waist” (D/W).
- b. “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 cells perimeter divided by the minimum distance between any two point of the cell boundary (waist) is less than “max_dist_over_waist” then 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.

- c. min pixels per cell.
- d. 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.

This parameter is useful to limit 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, 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.

- e. “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 brightfield 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.

Brightfield images taken slightly out of focus may do better with higher values (ie, 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.

- f. “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 timepoints for them to be considered the same cell. The default value is 0.2.

- g. “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 brightfield 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 brightfield image to match the cells in the fluorescence images. This feature is useful when cells move slightly between image acquisitions.

After selecting values for these options, click “Test Cell-ID”. Cell-ID will do a full processing of the whole set corresponding to that brightfield image and it will display the output files on the main screen (Figure 14.18.4). It will also create an output_all.txt file with the numerical data to be analyzed. OK to return to the main menu.

- 18 In the brightfield image, check if Cell-ID appropriately found the majority of the cells. Depending on how “clean” the background of the brightfield image is, Cell-ID might also have found structures in the image that were “mistaken” as cells. These incorrectly called, “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 (Figure 14.18.5).
- 19 Now 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”.
- 21 If the test was satisfactory, try it on another set.
- 22 When you are convinced that 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. Clicking on the “Run” button will process the whole experiment. Cell-ID will have created 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 used input files, and the generated output files.
- 23 Now you are ready to analyze the data.

Analyze data with R

Loading data into 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 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.

- 24 Open R and load the R package “Rcell”. You can do this by typing **require(“Rcell”)** in the R console, or from the *packages -> load package* menu if you are using an R-GUI.

- 25 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.

- 26 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 1) and creates a few extra variables (Table 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. Non-fluorescent related variables are not renamed. For example, a.tot, pos and t.frame remain as such (Table 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:

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

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). You can minimize these errors by adjusting the program parameters (step 17), but usually some badly found cells remain. Furthermore, the program cannot discriminate out of focus and dead cells.

- 27 The R package allows, among other things, to filter out unwanted cells from your data set. This first filtering step is intended to eliminate from your dataset, spurious, out of focus and dead cells. If you are 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 appears in a particular set of timepoints (for example, all time points after the first five (step 28).
- Inspect the output images created by Cell-ID and the histograms plotted by the **show.hist** function provided in the package.
  - 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, such as 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. You can do additional cuts in whichever variable you prefer. If the variable distribution is not plotted by **show.hist**, you can plot it with **chist**, as shown below. Select the cut limits and apply them using the **QC.filter** function.
- e. If you selected the "treat bright as fluorescence also" option when running Cell-ID, you may use the intensity of the brightfield image in the analysis. Out of focus cells usually show a high intensity in the brightfield image, and thus can easily be removed including in the cut `f.tot/a.tot<[your chosen value]` for this channel.

A quality control cut might look like this

```
X<-show.hist(X) #quick overview of some variables distribution
```

```
X<-QC.filter(X,fft.stat<0.3) #filter the cells by circularity
```

```
X<-QC.filter(X, f.tot.y/a.tot-f.bg.y<100) # cut on fluorescence density of the YFP channel
```

```
X<-QC.filter(X, a.tot>100 & a.tot<800) #upper and lower cut on cell area.
```

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)**).

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

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, or moved so much during the experiment that Cell-ID assigned it a different Id half way through the time course. Beware that the total number of frames in which a cell appears is calculated after the quality control filter is applied. For this reason you cannot 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)**).

- 29** Verify that quality control and cell selection were adequate using the **show.img** function.

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

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

Basic plotting of data—Once the data is loaded in R and filtered, you can plot the data. 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.

- 30** Plot the YFP total fluorescence vs 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 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 functions, for example **chist** and **cplotmeans**, that operate over **hist** and **plotmeans** (the latter belonging to the "gplots" package), respectively. Note that the postfix of f.tot will vary with the channel.

- 31** 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.

```
cplot(X,f.tot.y~t.frame,pos=1) #plots the total fluorescence over time for
position 1
```

```
cplot(X,f.tot.y~t.frame,pos=1,filter=(a.tot>400) ) #plots cells bigger than 400
pixels only
```

```
#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

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

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.

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, for example, 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.

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

```
X<-append.pos.var(X,treat,"treatment") # appends (adds to the  
dataset) the new "treatment" variable
```

- 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.

Measuring 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 previous variables are be called 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 should be cytoplasmic.

Expressions 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. $(f.m0 - a.m0 * f.bg) / (f.tot - a.tot * f.bg)$.

```
cplot (X,(f.m0.y-a.m0*f.bg.y~t.frame),pos=3) # plots "a  
vs time point" for every cell in Position 3.
```

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

Measuring nuclear fraction of fluorescence

- 36** Cell-ID calculates both the total fluorescence associated with a cell (f.tot), and the nuclear fluorescence (f.nucl) for each channel (f.tot.y and f.nucl.y, for YFP, for example). To calculate the nuclear fraction for a given channel, do the ratio of nuclear to total fluorescence ((f.nucl-a.nucl*[auto])/(f.tot-[auto]*a.tot)). "auto" is not a variable available directly 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.

Alternate Protocol

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/increased speed, reduced donor fluorophore photobleaching, and reduced phototoxicity.

The protocol below refers 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 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 partner 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 brightfield image.

We have not measured FRET in particular locations using a separate color (e.g., nuclearly localized RFP to identify the nucleus) using 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

Hardware

Fluorescence microscope: Same as in the basic protocol, except that you must remove the emission filter from the cube used to excite the donor fluorophore (for CFP/YFP FRET, remove the emission filter from the CFP cube). This is referred to as the FRET cube.

Image splitter: This protocol describes procedures to analyze images that are split horizontally into top and bottom sub-images. This requires an image splitter with an appropriate dichroic mirror and top and bottom emission filters. For CFP/YFP FRET, you can use, for example, 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.

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. It is recommended that you 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. This orientation will be assumed for the rest of this protocol. To test, take a test brightfield 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 brightfield image with the FRET (modified CFP) cube. Now both the top and bottom half should have an image.*
- 3 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

- 4 Follow the basic protocol for preparing cell samples.

Acquire images

- 5 Reducing autofluorescence. This procedure can be useful in any number of other applications. *Yeast contain various fluorescent compounds, which together constitute cellular autofluorescence, which 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.*

To remove the rapidly photobleachable autofluorescence, perform a number of “pre-bleaching” image sets. The number can be established 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.

- 6 Follow the basic protocol for determining exposure settings. Keep in mind that each timepoint for each image field requires two exposures, a FRET exposure and an acceptor fluorophore exposure.
- 7 Acquire a brightfield image following the basic protocol. Use the FRET cube to ensure that the cells appear in both the top and bottom image panels. Label the image BF_Position[X].tif, where X is a number. Make sure all of numbers [X] have the same number of digits; for example, if you have between 1 and 99 images, label them 01, 02, 03...; if you have between 1 and 999 images, label them 001,002,003... etc. If you are taking multiple timepoints 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.
- 8 Acquire an acceptor excitation image using the YFP cube. Name it YFP_Position[X].tif or YFP_Position[X]_time[Y].tif, depending on if you are taking multiple timepoints. The fluorescence will only show on the bottom half of the image.
- 9 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.
- 10 Repeat 6 to 8 for each time point and image field in which you want to measure FRET.
- 11 After collecting all images for all positions and all timepoints, place all images into the same folder.

Process images with Cell-ID

- 12 Proceed to load images as described in the basic protocol. For a typical nuclear CFP/YFP FRET experiment, for example, set the following parameter values given filenames as described above:
  - a. Position token: Position
  - b. Time token: time
  - c. sep. character: _
  - d. brightfield: BF
  - e. Fluorescent: FP
  - f. nuclear image: YFP

Other options set as described in the basic protocol.

Note: Flat is generally ignored. The typical FRET metrics are ratiometric in nature (i.e. takes 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.

- 13 Setup segmentation according to 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 cut during later analysis.
- 14 Test to see if Cell-ID is identifying cells in the split brightfield image properly, as outlined 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.

- 15 Start batch calculation as described in step 22 of the Basic Protocol.

Analyze data with R

When performing split image FRET data extraction, Cell-ID outputs essentially the same variables as it does for each color in the basic protocol, except that in addition to a color identifier for the fluorescent related variables (see table 1), there is also the sub-image identifier for all variables (except pos, t.frame and cellID) . When the output files are loaded in R, the variables are renamed taking into account the channel and sub-image type. 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 non fluorescent 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).

- 16 Proceed with data analysis as outlined in the Basic Protocol to examine basic cell properties (total fluorescence, area, etc.).

Support Protocol 1

Obtaining and installing Cell-ID and R

R and Rcell

1. Download and install the latest binary distribution of R appropriate for your operating system, from the download section of the R-project webpage: www.r-project.org.
2. Download and install the latest binary release of ImageMagic for your operating system from www.imagemagick.org.
3. Download and install GTK+ from 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")
source("http://bioconductor.org/biocLite.R")
biocLite("EBImage")
install.packages("C:/.../Rcell-[xxx].zip", repos=NULL)
```

Alternatively, you can install them using the Packages->Install Packages menu of the R GUI.

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, you can download it from <http://www.bioconductor.org/packages/release/Software.html>, where you can find 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

at the prompt of a new X11 terminal:

```
R CMD INSTALL Rcell-[xxx].zip
```

Cell-ID and VCell—Source code for Cell-ID and VCell can be downloaded from the Cell-ID website at <http://lbms.df.uba.ar>, along with required dependences and installation guides. An installation wizard for windows is also available.

Support Protocol 2

Preparing yeast and mammalian cells for imaging

The exact culture conditions depend on the experiment. Below are standard procedures to obtain an exponentially growing yeast population or mammalian population that will display low autofluorescence.

For yeast

1. Two days before the experiment, Grow yeast overnight in the appropriate medium according with the required auxotrophies.
2. In the morning, dilute cells to 0.05 OD₆₀₀ in fresh medium. Grow for at least 6 hours. The goal is to shift the culture to exponential growth. During this time, prepare 4 glass tubes with 5 ml of synthetic medium supplemented all amino acids (except for those omissions required to select for plasmids, if any) and with 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.

3. Measure the OD₆₀₀ in the afternoon and dilute the sample to OD₆₀₀=1 × 10⁻³ in the first tube. Perform serial dilutions 1/10 in the three remaining tubes. The goal is to have one of the tubes at OD=0.1 the next morning. Put the tubes in a rotating wheel or shaker at 30C.

Assuming a normal doubling time of 90 minutes, 15 hours 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. (to image fixed cells go to step 12).

4. The morning of the experiment, or the day before, add 100 µl of 1mg/ml PBS solution of concanavalin A (Sigma C7275) to multiwell glass bottom plates. Incubate for at least 30 minutes, up to overnight at room temperature.
5. Wash three times with water.

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

6. Add to the cells 200 μM Ca^{2+} and 100 μM Mn^{2+} . This will improve the interaction of concanavalin A with the cell wall sugars.
7. Add 100 μl of yeast $\text{OD}_{600} = 0.1$ to the wells. Wait 10 minutes and wash unbound cells with fresh medium three times.
8. 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.

9. To stimulate cells, pipette 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.

Steps 12 to 16 only for fixed cell imaging

10. Perform your experiment as usual, and take samples at the desired timepoints.

11. For every 1 ml of sample, add 1 ml of ice cold 4% paraformaldehyde (pH7 with PBS) and incubate for 1 hour on ice.

12. Add 50 μl 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.

13. Spin 20 seconds at top speed in a microfuge. Discard the supernatant and add 500 μl of PBS.

14. Repeat two more times.

Yeast are ready to load on the imaging plate.

For mammalian cells

1. Add 100 μl of 1mg/ml polylysine solution (Sigma P4707) to each of the wells of a 96 well plate and incubate for at least 30 minutes at room temperature. Wash polylysine with PBS. The plates are good for a few days stored in at 4C.
2. Split your culture one or two days before the experiment, so that the cells are actively growing.
3. Imaging fixed cells. This protocol works for many cell types, including HEK293, 3T3, HeLa.
  - a. Perform your experiment as usual. To prepare the samples for images.
  - b. Remove medium and add ice cold paraformaldehyde 2% (prepared in PBS, making sure the pH is 7). Incubate 1 hour on ice.
  - c. Remove paraformaldehyde and wash cells three times with PBS.
  - d. Trypsinize cells as usual, wash trypsinized cells by centrifugation and resuspension in medium, and pipette 100 μl into the pre-coated wells.

Cells are ready for imaging.

4. (alternate) Live imaging.

Special considerations.

- Extra equipment: temperature and gas controlled incubator mounted on the microscope.
  - 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, such as lymphocytes, but does not with cells with extensive spreading and processes, such as epithelial or neuronal cells. Images of cells that spread on a 2D 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, such as medium without phenol red, or use medium diluted to half with PBS.
- a. 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.
  - b. Set up your plate in the microscope, making sure that the plate on the stage 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.
  - c. To stimulate cells, pipette 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.

Support Protocol 3

Calculating nuclear and plasma membrane CFP-YFP FRET using split images

FRET calculation—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).

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):

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

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 one wants to calculate FRET in, such as the membrane, nucleus, or for the whole cell (see below for more details).

These three measures are background and “crosstalk” corrected as follows:

$$CY = CY_{\text{obs}} - B_{CY} - A_{CC \rightarrow CY} (CC_{\text{obs}} - B_{CC})$$

$$CC = CC_{\text{obs}} - B_{CC} - A_{CY \rightarrow CC} (CY_{\text{obs}} - B_{CY}), A_{CY \rightarrow CC} \sim 0 \text{ (see below), thus, } CC = CC_{\text{obs}} - B_{CC}$$

$$YY = YY_{obs} - B_{YY}$$

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. For nuclear FRET, a good estimate of these quantities 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 setup. You can define up to four radii for nuclear quantification when processing the images with Cell-ID. 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.

$A_{CC \rightarrow CY}$ and $A_{CY \rightarrow CC}$ are the “crosstalk” coefficients of CFP into the YFP channel and of YFP into the CFP channel (see below).

2. Performing crosstalk corrections.

There is significant CFP protein emission crosstalk into the YFP emission channel due to its broad emission spectrum (“spectral bleedthrough”); 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 crosstalk of YFP protein into the CFP emission channel is usually negligible.

$A_{CC \rightarrow CY}$ is the conversion factor that allows to calculate how much signal one should expect to observe in the YFP channel due to CFP protein emission. The exact magnitude of the crosstalk coefficients $A_{CC \rightarrow CY}$ and $A_{CY \rightarrow CC}$ depends on the particular illumination and filter cubes used.

To calculate the $A_{CC \rightarrow CY}$ coefficient, use purified CFP. Take the ratio of YFP emission channel signal to CFP emission channel signal in a split image obtained with the FRET cube using a solution of pure CFP protein. For the image splitter configured as outlined above, is the ratio of the bottom image signal to the top image signal. Using recombinant CFP we obtained 0.99. With the optics outlined here, $A_{CY \rightarrow CC}$ is negligible.

Measuring Nuclear FRET in Yeast

1. When processing these images, select 3, 6 and 7 for the radius in the “nucleus size” option in the “Image Setup” window, which will be assigned to the variables $f.nucl$, $f.nucl2$ and $f.nucl3$, respectively. These variables will be used below to calculate the background cellular fluorescence in each emission channel.
2. To measure nuclear FRET, use the following quantities in the N_{FRET} equation above.

a. CC:

$$CC_{obs} = f.nucl.cu/a.nucl.u$$

$$B_{CC} = (f.nucl3.cu - f.nucl2.cu)/(a.nucl3.u - a.nucl6.u)$$

b. CY:

$$CY_{obs} = f.nucl.cl/a.nucl.l$$

$$B_{CY} = (f.nucl3.cl - f.nucl2.cl) / (a.nucl3.l - a.nucl2.l)$$

$$A_{CC \rightarrow CY} = 0.99 \text{ (see above)}$$

c. YY:

$$YY_{obs} = f.nucl.yl / a.nucl.l$$

$$B_{YY} = (f.nucl3.yl - f.nucl2.yl) / (a.nucl3.l - a.nucl2.l)$$

Measuring plasma membrane FRET in Yeast

1. In the image setup window select the nucleus quantification radius used above and the "force nucleus to center" option, which will cause the program to calculate the fluorescence of concentric disks centered in the cells geometric center. Note you do not need a nuclear-tagged fluorescence image.
2. To measure membrane FRET, use the following quantities in the N_{FRET} equation above.

a. CC:

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

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

b. CY:

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

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

$$A_{CC \rightarrow CY} = 0.99 \text{ (see above)}$$

c. YY:

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

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

COMMENTARY

Background information

Single cell measurements 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 have identified other processes and genes that account for and control the bulk of the variation (Colman-Lerner et al., 2005).

One means to collect single cell data is flow cytometry. The instruments interrogate cells as they pass individually through an apparatus that excites fluorophores with a laser and collects the emitted light. These instruments are powerful (Bonetta, 2005; Shapiro, 2003) 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, to excite cells and collect emitted light for long times, and to 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, (i.e., "Metamorph", Molecular Devices Corporation, Sunnyvale, California (Gonzales and Woods, 2002; Inoue and Inoue, 1986; Schnapp, 1986) and "ImagePro", Media Cybernetics, Inc., Silver Springs Maryland), 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. 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 (Goldberg et al., 2005; Swedlow et al., 2003), Image J, a Java-based package of microscope image analysis tools (Abramoff et al., 2004), and CellProfiler (*UNIT 14.17*), initially developed to help analyze images of cells arrayed on solid supports (A. Carpenter, pers. comm.).

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 tuned to work with yeast cells, it 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 (such as the BD LSRII), and 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, such as phase contrast, differential interference contrast, or even fluorescence images, although for the last case our work suggests that a pre-processing step might be necessary to determine the boundaries of the fluorescent regions.

Critical parameters

Acquisition

Z control and the brightfield image: Critical to find the dark ring in a defocused image consistently. Image to image differences in Z position in a time course, for example, 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: Critical for imaging cells with large differences in FP levels. Sensitivity: important for reducing photobleaching when measuring time courses.

Objective: High numerical aperture: To image low levels of light

Exposure time: To balance photobleaching and phototoxicity with higher signal-to-noise ratio.

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

Troubleshooting

If cells are not identified, try defocusing the brightfield 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 timecourse.

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Figure 14.18.1.

Example of cells yeast and mammalian cells processed by Cell-ID.

Top. 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 identified each one with and ID and traced their borders correctly.

Bottom. HEK293 cells fixed, trypsinized and imaged as described in the protocol, before (left) and after (right) Cell-ID located them in the image.

Figure 14.18.2.

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

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, on the left is the directory tree, with BF_Position04.tif selected. That image is shown in the central window.

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 called 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.

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 plot of the output of a composition of two calls to **cplotmeans**. The bottom window shows the R console, with executed commands.

Table 14.18.1

Cell-ID output variables

	Variable name	Description
<i>General measurements</i>		
1	cellID	The cell identification number. In fret experiments the cells in the upper and lower part of the split image are differentiated by an offset this value.
2	t.frame	The 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	The 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	The X coordinate of the centroid of the cell
5	ypos	The Y coordinate of the centroid of the cell
6	f.tot	The sum of the fluorescence image for all the pixels found in that cell
7	a.tot	The area of the cell in pixels
9	fft.stat	This statistic is derived from the one-dimensional fast-fourier-transform (FFT) of the function: radius vs angle, where 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.
10	perim	The circumference of the cell in pixel units
11	maj.axis	The length of the major axis in pixel units
12	min.axis	The length of the minor in pixel units.
15	flag	The "flag". It indicates the "image type." Thus if there are for example 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.
16	rot.vol	The volume of rotation of the cell around its major axis
17	con.vol	The volume of the cell as determined by the "conical volume" method
18	a.vacuole	The vacuole area (calculated from for darker regions of fluorescence within the cell)
19	f.vacuole	The vacuole fluorescence, respectively (calculated from for darker regions of fluorescence within the cell)
20	f.bg	The fluorescence background level. This quantity is the mode of the distribution of all fluorescence pixels that are not associated with any cell.
<i>To calculate membrane proximal fluorescence (for relocation experiments):</i>		
21	f.tot.p1	The fluorescence and area of all the pixels that are interior to the boundary that is one pixel wider than the cell boundary. These numbers thus include the original cell plus an annular region one pixel around the outside of the cell.
22	a.tot.p1	The area of all the pixels that are interior to the boundary that is one pixel wider than the cell boundary. These numbers thus include the original cell plus an annular region one pixel around the outside of the cell.
23	f.tot.m1	The fluorescence of all pixels that are interior to the boundary that is one pixel smaller than the cell boundary.
24	a.tot.m1	The area of all pixels that are interior to the boundary that is one pixel smaller than the cell boundary.
25	f.tot.m2	The fluorescence of all pixels that are interior to the boundary that is two pixels smaller than the cell boundary.
26	a.tot.m2	The area of all pixels that are interior to the boundary that is two pixels

	Variable name	Description
27	f.tot.m3	smaller than the cell boundary. The fluorescence of all pixels that are interior to the boundary that is three pixels smaller than the cell boundary.
<i>Information obtained from the "nuclear image" type. The variables contain the area and fluorescence of concentric disks of user defined radius.</i>		
28	f.nucl	The total fluorescence of the found nucleus. To find the nucleus Cell-ID moves a disc of given radius (default to 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 user defined radius (default to three pixels). If there is a nuclear tagged image, it uses it to find the nucleus and returns the fluorescence for every channel. If there is no such image it searches for the nucleus in every channel and returns the found fluorescence in each channel.
29	a.nucl	The area of the found nucleus
30	xnucl	The X coordinate of the nucleus center
31	ynucl	The Y coordinate of the nucleus center
32	f.nucl2 a.nucl2	Same as f.nucl and a.nucl for an other user defined radius.
33	f.nucl3 a.nucl3	Same as f.nucl and a.nucl for an other user defined radius.
34	f.nucl4 a.nucl4	Same as f.nucl and a.nucl for an other user defined radius.
<i>More background information</i>		
35	f.local.bg	A measure of the background level at pixels located 5 radial pixels further out than the cell boundary. It is thus a measure of the local fluorescence background level. The background level is the average fluorescence per pixel. For this calculation only pixels along the annular boundary that are NOT associated with ANY cell are included. The background level here is the mean of the pixels.
36	local.bg.num	The number of pixels used in the background calculation for local.bg
37	local.num	The total number of pixels along the annular region, including all pixels (which means pixels associated with cells and pixels not associated with any cell).
38	f.local2.bg	The same information as the previous 3 columns but using the background
39	local2.bg.num	level at pixels located x radial pixels outward of the cell boundary, where x
40	local2.num	is one half of the minor axis of the cell.
<i>More volume measurements</i>		
41	a.surf	The surface area as calculated from the "union of spheres" method .
42	sphere.vol	The volume as measured b the "union of spheres" method

Table 14.18.2

Variables calculated from, and appended to Cell-ID output by the Rcell package

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