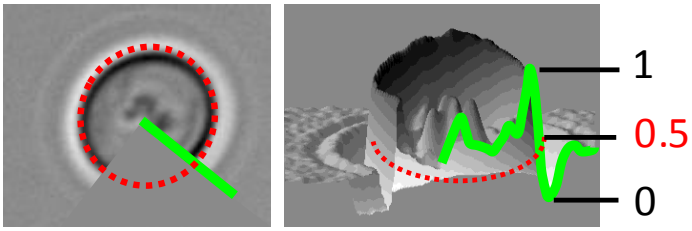
BudJ v7.7

Overview

BudJ has been designed to obtain dimensions and fluorescent data of budding yeast cells. Specific procedures using object libraries supplied in ImageJ (Wayne Rasband, NIH) have been written to detect cell boundaries as pixels markedly darker compared to both the surrounding background and the cell interior. The algorithm first obtains a densitometric profile along a radial axis from a seed point within the cell (initially provided by the user) to find two consecutive maximum and minimum pixel values produced around the cell boundary, which is arbitrarily established at an intermediate pixel having their arithmetic grayscale mean as value (see figure below).



Then, cell boundary pixels are iteratively defined by full rotation of the radial axis and a preliminary ellipse object is fitted. Outliers are first eliminated by their distance to the center of the ellipse when it is too large compared to average. Second, outliers are eliminated if the difference in gray values at the edge is too small. Third, more outliers are eliminated if the radial difference to neighbors is too large. Once outliers have been removed, an ellipse is fitted to the obtained boundary pixel array, and major and minor axes are used to calculate the cell volume assuming a prolate as shape. The same cell is followed through consecutive time-lapse images by using the center of the ellipse as seed point to obtain radial profiles in the following time point image.

Since BudJ v7, user-assisted and self-learning modes have been implemented to improve cell tracking in time-lapse experiments, particularly in highly crowded cell areas. In user-assisted mode, the ellipse or oval selection tools are used to indicate BudJ the approximate limits of the cell. In self-learning mode, after the user accurately marks the limits of the cell in the first time frame with the ellipse selection tool, the program locates the cell in subsequent time frames using specific pixel information at the cell edge from the preceding frame.

Once the cell has been delimited, BudJ will analyze fluorescence data from all pixels within the cell and provide with different sort of basic statistics. In addition, the intracellular distribution of brighter pixels can be analyzed by three different feature types: cluster, foci and patch. Finally, if fluorescence data from two channels is provided, a colocalization analysis of the whole cell or selected features can be performed.

Installation

Create a BudJ folder within the plugins folder of ImageJ, copy BudJ files (BudJ_class, CellBJ.class, EllipseBJ.class, PointRoiBJ.class, ScrollbarBJ.class) into the BudJ folder. Make sure that you have the JAMA library installed (jama_x.x.x.jar file in the jars folder). Once ImageJ is restarted, the BudJ folder will appear under the plugins menu option of ImageJ.

Procedures

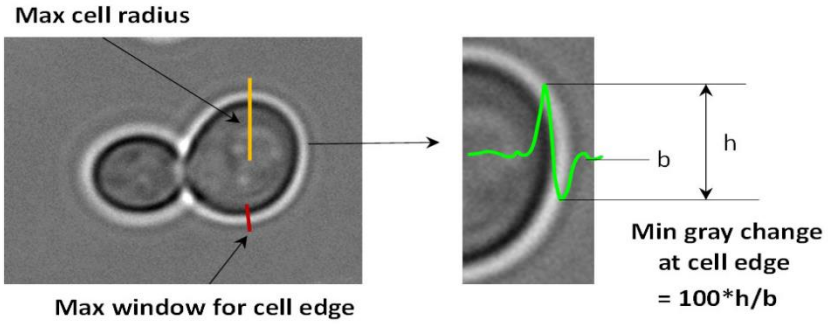
1. Open image and check image properties

Open stack image in ImageJ. The program is designed to determine the cell limits in **bright field** (BF) images. DIC images are **NOT** appropriate to detect the optical cell limit with BudJ. Time-lapse 16-bit or 8-bit hyperstacks with up to three channels (BF, FL1 and FL2) can be analyzed. BudJ also deals with hyperstacks that contain a z dimension. These stacks are a must when autofocus is not available or not precise enough to ensure a proper focus of the cell during the whole time-lapse experiment. FL may be obtained from the best focused z slice on the BF image or from a z projection with the maximum or average pixel values.

2. Run BudJ and select parameters

Execute BudJ from the plugin menu and define required parameters in the open dialog box:

- **BF channel** – The channel number of the bright field image.
- **Scale** – Check that the scale factor is correct, and modify if needed. For best results, images should have a **scale factor <= 0.1 µm/pixel**.
- **Max cell radius** – The maximum radius for cells in the experiment (4 µm as default). This value helps BudJ to discard wrongly identified cell limits.
- **Max window for cell edge** – Radial window where the cell edge is detected (1 µm as default).
- **Min gray change at cell edge** – Percentage difference between the maximum and minimum pixel values relative to the background pixel value (20% as default). For setting the minimal gray change to detect the cell edge in your bright-field images you should obtain a profile across an average cell and estimate the difference of the brightest and dimmest pixel in the sigmoidal profile around the edge of the cell. This difference has to be made relative to the background outside cells. Furthermore, brightest and dimmest pixels in the sigmoidal profile must be closer than **Max window at cell edge**.

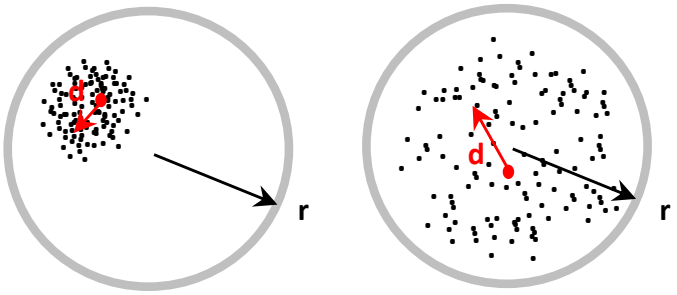


These 3 parameters are very important for BudJ to work properly !!!

- **Radial variation allowed** – Only used in time-lapse stacks, where it sets the percentage difference allowed in cell radius between consecutive time points. This parameter helps BudJ to discard a cell wrongly assigned in the following time frame.

Up to two fluorescence channels can be analyzed with additional parameters:

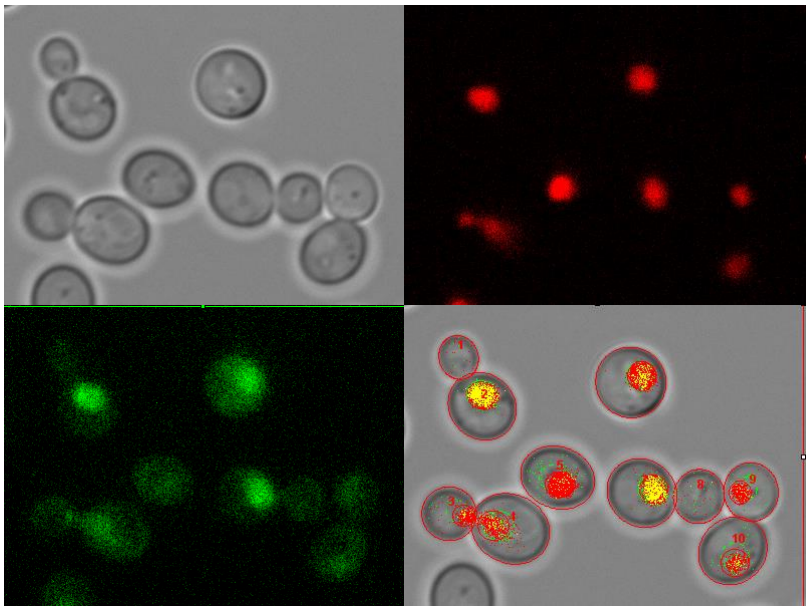
- **FL1/2 channel** – Fluorescence (FL) channel number in image stack. Enter 0 to disable.
- **FL1/2 data** – Enter the type of FL data to obtain from cells
  - **FL stats** – Mean FL and standard deviation of pixels within the cell. The modal value of the entire image is also provided as a first guess of the background FL value, but this approximation will likely fail in very crowded images. Please always make sure of selecting the right background value for further calculations with fluorescence data.
  - **FL stats & freq** – FL stats plus pixel frequencies by their FL value (0 to 255).
  - **Cluster index** – FL stats plus an index of pixel clustering. First, pixels within the cell are selected as being brighter relative to a **Min FL change at cluster**, which is the percentage difference relative to the median FL of pixels within the cell (30% as default). The brightest selected pixels are used to calculate a FL-weighted gravity center and, then, the FL-weighted mean distance of selected pixels to the gravity center is made relative to 8r/15, the theoretical mean distance to the center from random points in a sphere projected to a circle with radius r.



$$\text{nuclear index} = \frac{8r/15}{\text{mean}(d)}$$

d = pixel distance to gravity center ●
r = projected circle radius

Thus, the cluster index is inversely related to the mean distance of the brightest pixels to their gravity center, so the closer the pixels the higher the index. This is useful to estimate the level of a fluorescent protein in a single globular compartment of the cell such as the nucleus. A **Cluster area** must be provided by the user as a percentage of the projected area of the cell (17% as default, which would correspond to the nuclear compartment). Mean FL and standard deviation of pixels within the specified cluster area are given. If the number of selected pixels is lower than 20% of those corresponding to the specified cluster area, then the cluster index is set to 1, and a central area of the cell is chosen to obtain fluorescence data. As an option, BudJ is able to select only **side-by-side** pixels in the final cluster. In this case, the cluster index value becomes irrelevant and it is assigned -1 as value.



Example of Cluster analysis applied to nuclear colocalization of two proteins

- **Foci data** – FL stats plus data of bright pixels forming foci within the cell. Pixel groups are selected as foci if they are brighter than a **global** threshold set by **Min FL change at foci**, which is the percentage difference relative to the median FL of all pixels (30% as default). Only pixels with a FL value above this threshold are selected as foci if pixel area is larger than **Min foci area**. As an option, BudJ uses **local** thresholds that are obtained from small circular regions around each pixel defined by the entered **Local threshold radius**. In some instances, it is useful to impose **both** thresholds. Checking **Single Foci Data** will force BudJ to identify individual foci, but this may be a very lengthy process if cells contain a large number of pixels in foci. Unchecking **Single Foci Data** will restrict data to the total area and the mean FL value in foci, but these info may be sufficient in most cases.
- **Patch data** – FL stats plus data of bright patches within the cell. Patches are defined by **Patch width** in pixels and are selected if they are brighter than **Min FL change at patch,** which is the percentage difference relative to the mean FL of pixels surrounding the patch (30% as default). Only patches with a mean FL value above this threshold are selected.

- **Feature color** – Select the preferred color for displaying features on image.
- **FL1<->FL2 colocalization** – Colocalization analysis.
- **FL analysis in Z stack** – Chose one of the following options for z stacks
  - **Best focused z** –FL data are obtained from the best-focused BF z slice.
  - **MAX projection** – FL data are obtained from a z projection by the maximum pixel value method.
  - **MEAN projection** – FL data are obtained from a z projection by the average pixel value method.

Parameters for time-lapse analysis:

- **Time unit** – Time units used.
- **Time interval** – Time interval between time frames in image hyperstack.
- **Time points to analyze** – Time points to be analyzed for each selected cell.
- **Time direction** - Time points can be analyzed in a forward or reverse mode.

3. Select cells to be analyzed

Select the **POINT** or **HAND** tools in the ImageJ main menu and click on the cell to be analyzed in **Click and Get** mode, which is the default mode for identifying cell limits. BudJ will search for the cell edge as explained above and draw an outline of the cell at the clicked time frame and all consecutive time points. If BF parameters are not correctly set or cell is not properly focused cells will not be detected and BudJ will display a "Not found" message. Use the **BF Settings** button (see below) and check all BF parameters as explained above. If a cell changes its position in consecutive time frames BudJ will also display a "Not found" message. You can select the cell by clicking it again to resume the analysis, or press the **New** button (see below) to terminate the analysis on that cell, and selected data for output will be added to the results table/s. Cursor keys scroll through consecutive time frames (or z slices) in the image stack to easily verify that the cell is properly delimited in consecutive time frames. In cell crowded or poor contrast images, you may select the **User Assisted** or **Self Learning** modes to assist BudJ in correctly delimiting the cell on subsequent time frames in mode.
In a separate window you will find the following option boxes or buttons:

- **Click and Get / User Assisted / Self Learning** – Selects the specified analysis mode.
    - **Click and Get:** Click the cell close to its center with POINT or HAND tools. BudJ will search for the cell edge as explained above.
    - **User Assisted:** Approximate the cell limits with the ELLIPSE or OVAL tools. BudJ will search for the cell edge only around the selected ROI limits.
    - **Self Learning:** Accurately mark the cell limits in the first frame with ELLIPSE or OVAL tools. BudJ will use pixel information around the marked cell limits to find the cell edge in consecutive time frames.
- **1 to 4** – Stores cell in group 1, 2, 3, or 4.
- **New** – Only used in time-lapse stacks; terminates analysis of current cell and outputs obtained data. As a convenient shortcut key, pressing the spacebar has the same effect.
- **Single Frame / Time Lapse** – Sets frame by frame or whole time-lapse analysis. Very useful when editing cell selection in time-lapse stacks.
- **Remove Last** – Removes last clicked cell from the analysis or, in time-lapse stacks, removes from analysis all time frames starting with the currently selected frame.
- **Hide / Show Overlay** – Hides or shows the overlay with cell data.
- **FL Settings** – Lets you modify FL parameters and update detected features in last clicked cell.
- **BF Settings** – Lets you adjust BF parameters to improve cell limit detection.
- **Reset** – Re-initiates BudJ on current image and lets you change all parameters.
- **Help** – Displays a brief help message.
- **Exit** – Closes image and shuts down BudJ.

4. Data output

The following data are added to the results table/s:

- **Cell group** –Group of cells as selected when the cell is clicked (see below).
- **Cell #** – Cell number in the selected group.
- **Time frame #** – Time frame number in the time-lapse stack.
- **Centroid X** – X coordinate of the center of the cell ellipse in image.
- **Centroid Y** – Y coordinate of the center of the cell ellipse in image.
- **Major radius** – Major radius of the cell ellipse.
- **Minor radius** – Minor radius of the cell ellipse.
- **Ellipse angle** – Angle of the cell major diameter relative to the X axis of the image.
- **Edge** – Mean distance between pixels with maximum and minimum pixel values at the cell boundary. Provides a relative index of focus degree of the cell.
- **Best focused slice** – If a z stack is being analyzed, BudJ selects the z slice with the lowest **Edge** value, and obtains all selected FL data from this z slice.
- **Cell volume** – Volume of the cell as deduced from a prolate object with **Major radius** and **Minor radius** obtained by BudJ from cell boundary pixels.
- **FL1/2 FL stats** – Mean FL and standard deviation of pixels within the cell.
- **FL1/2 distribution** – Pixel distribution as a function of FL values.
- **FL1/2 cluster index** – Cluster index at the chosen FL.
- **FL1/2 cluster FL stats** – Mean FL and standard deviation of pixels within the estimated cluster area.
- **FL1/2 foci** – Number of foci found.
- **FL1/2 foci size** – Mean size and standard deviation of foci.
- **FL1/2 foci FL stats** – Mean FL and standard deviation of foci.
- **FL1/2 patch #** – Number of patches found.
- **FL1/2 patch FL** – Mean FL of patches found.

If colocalization analysis is performed the following data are added to results table/s:

- **PCC** –Pearson's correlation coefficient for all cell pixel values in FL1 and FL2.
- **MCC FL1/2** – Mander's colocalization coefficients for cell pixel values above median in FL1 and FL2.
- **PO FL1/2** – Percentage overlap of cell pixel values above median in FL1 and FL2.
- **PFO FL1/2** – PO of feature (cluster, foci, patches) pixel values in FL1 and FL2.
- **FL1/2 mean in FL2/1** – Features in one FL channel are also provided with FL data from the other channel.

  Users are suggested to use two-sample t tests for comparing PCCs or MCCs from different conditions to obtain a p value (see Dunn et al. 2011. A practical guide to evaluating colocalization in biological microscopy. Am J Physiol Cell Physiol 300:C723-42).

  Percentage overlap data may be compared using binomial statistics.

5. Acknowledgements

BudJ is freeware. If you use this software for a publication, I would appreciate that you cite it as **BudJ: an ImageJ plugin to analyze fluorescence microscopy images of budding yeast cells** (http://www.ibmb.csic.es/home/maldea). This software is provided as is without any warranties,

but I do my best to make a well tested and good package, so bug reports and suggestions will be greatly appreciated.

Martí Aldea
IBMB-CSIC
Baldiri Reixac 15
08028 Barcelona
Catalonia, Spain