## Introduction

 The cell tracking software package provides a graphical user interface (GUI) to extract quantitative time series data for single cells from movies obtained from a fluorescent microscopy experiment. The user supplies raw focused and out of focus bright field (BF and BFOOF, respectively ) \*.TIF or \*.STK image files which are then segmented to create a cell region mask for tracking and application to fluorescent images (alternatively, a pre-segmented mask can be supplied). These cell regions are tracked through time, and mother-bud lineages are assigned. The user can then easily look through the movies in the GUI to curate cell ID and lineage assignments as well as segmentation errors. Finally, there is a built-in plotting tool for observing single cell or group traces, or the tracked data can be exported to a text file. The GUI can also be used to check segmentation of static image data (non-time series).

 The present version of the software has been designed for work with budding yeast, but should be flexible to suit other experiments as well. The segmentation module can be skipped (by providing a pre-segmented mask) or modified. Currently, the ProcessTimeSeries GUI can accept up to 3 fluorescent color movies and only 1 sub-region mask, but this will be more flexible in future releases. In addition, the colors must be called “RFP”, “YFP”, and “CFP” and will appear as red, yellow, and blue in the GUI, but this is purely superficial and has no quantitative effect on the analysis. Likewise, the sub-region mask must be called “N” (for “Nuclear”, with the remaining portion of the cell region called “Cytoplasm”), but this has no quantitative effect.

## Workflow

1. Obtain fluorescent microscopy movies from an experiment.
2. Pre-process data for tracking using the FormatData GUI. Includes image registration, cell segmentation, and sub-region mask capabilities.
3. Track cells and lineages through time with ProcessTimeSeries GUI, and curate ID and lineage assignments.
4. Select single cells or groups and plot various measurements using the GeneratePlots GUI accessible from the ProcessTimeSeries GUI.
5. Export tracked data to a \*.txt file for further analysis.

## FormatData GUI

 This GUI assists the user in setting up the data for processing with the ProcessTimeSeries GUI. Here, the different image channels are specified: BF (required), BFOOF (required for segmentation), the cell mask (required), fluorescent colors (optional, up to 3), and a sub-region mask (optional, up to 1). The GUI then formats the data according to the selected analysis type (Timelapse or Static) and creates a file in the specified save directory. This is the input for the ProcessTimeSeries GUI. This process must be repeated for each set of movies (BF, BFOOF, RFP for position 1, then BF, BFOOF, RFP for position 2, etc.).

1. Specify or browse to the folder containing the image files to set the data directory at the top of the GUI.
2. To the right, indicate which analysis type to perform. “Timelapse” will treat the image series as a time series (e.g., a movie of cells growing in a microfluidic chamber). “Static” will treat each image series as a set of snapshots taken from a population (e.g., multiple images taken at different positions on a single slide sample). For a time series analysis, image registration will correct for imprecise stage movements by shifting each image in the movies to minimize apparent cell movement within the frame. This greatly improves tracking (reducing manual track curation later), but will add random, “white noise” pixel values to fill in where images have been shifted at the border. Uncheck the box to skip registration.
3. Check the “Bright Field” box in the “Data Channels” panel. Click “Select” to the right to load your BF images. For \*.TIF files, select all BF image by holding the Shift key while clicking, then click open. For \*.STK files, just select the BF stack. If the images are successfully recognized, the file names will be listed in the popup menu to the right in the “Data Recognized” panel. (BF images are required. DIC or phase contrast images could be substituted if providing a pre-segmented cell region mask.)
4. Check the “Cell Mask” box. In the “Mask Source” panel, the first button toggles between “Segment BF” and “Supply Mask”. If supplying a pre-segmented mask, toggle to “Supply Mask”, use the “Select” button as in step 3 to load your mask files, listed in the popup to the right, and continue to step 6. If using the built-in segmentation module, toggle to “Segment BF” and continue to step 5. (A Cell Mask is required.)
5. If using the built-in segmentation module, check the “Bright Field (out of focus)” box and use the “Select” button as in step 3 to load your BFOOF files, listed in the popup to the right.(BFOOF obtained as BF -4μm relative to the focal plane at 63x works well.) See the note at the end of this section on the segmentation module.
6. If your data includes fluorescent movies, check the “Color Images” box. You can add up to 3 color channels using the “Add Color Data” panel. Enter the color name in the text edit box (in this release, limited to “RFP”, “YFP”, and “CFP”, but this is merely a label), then press “Add and Select” to load your color files as in step 3. The color name will be added to the list box on the left, and the file names will be added to the table on the right. If a mistake is made in loading the color files, select the color name in the list box on the left and click “Remove Color” to remove the files.
7. If you wish to have up to 1 additional sub-region mask based on one of the fluorescent colors (e.g., fluorescently labeled nucleus 🡪 nuclear region mask), check the “Color Mask” box. In the “Color Mask Source” panel, enter the sub-region mask name in the text edit box (in this release, limited to “N”). You may then push “Add and Select” to load pre-made sub-region mask files as in step 3, or you may choose a color from the popup menu in the “Color Mask Source” panel (requires the color to be added in step 6) and push “Add and Segment” to generate the sub-region mask using the built-in thresholding module applied to the selected color images. In each case, the color mask name and source will be displayed in the table to the left, with the relevant recognized file names appearing in the table on the right.
8. Specify or browse to the desired folder to be used as the save directory at the bottom of the GUI.
9. Press “Format Data” to process your data and create a \*.mat file in the specified folder. This file will serve as the input to the ProcessTimeSeries GUI.

Notes on image registration module

 Images are registered by computing the 2D cross-correlation between the BF image at time *t* and the next BF image at time *t*+1. Each image is padded at the boundaries at the boundaries with white noise ¼ the width of the image, and shifted to move the maximum of the 2D cross-correlation to the center. The same shift performed on the BF image, is performed on each other image channel to register all images similarly for time point *t*+1.

Notes on segmentation module (BFsegment.m)

 The included segmentation algorithm requires both a BF and BFOOF image. Our BFOOF images were obtained by moving the objective -4μm relative to the focal plane used for BF using the same acquisition settings as for BF. This switches the contrast of the cell walls from white-outside, black inside when focused, to black-outside, white-inside when out of focus. The algorithm first equalizes the brightness for each image series, and then compares the BF and BFOOF to find cell boundaries using a series of morphological operations. Regions comprising several cells are then segmented by watershedding. The current parameters work well for haploid and diploid budding yeast at 63x and 100x, but the BFsegment.m file can be customized to the user’s needs.

Notes on sub-region mask module (color2submask.m)

 The binary sub-region mask is generated by auto-thresholding the specified color image for light objects, raising this threshold by a defined parameter, and then binarizing the color image based on the new threshold. The binary sub-mask is then cleaned using several morphological operations. The color2submask.m file can be customized to suit the user’s needs.

## ProcessTimeSeries GUI

 This GUI accepts the file created by the FormatData GUI, filters regions, tabulates cell measurements for each region over all time points in each data channel (color, mask, etc.), tracks cell regions through time using the region centroid and a Matlab-adapted version of the IDL Particle Tracking software developed by David Grier, John Crocker, and Eric Weeks (available at <http://physics.georgetown.edu/matlab/code.html>), and assigns lineages. The user is then able to quickly and visually curate poorly segmented regions and incorrectly assigned cell IDs and lineages using a combination of GUI tools and shortcut keys. To facilitate this process, recurring IDs appear in yellow, while new IDs appear in pink if they are assigned as a bud, and green if no bud is assigned. An embedded graphing module allows plotting of selected single cell or group trajectories for various measurement combinations. Finally, selected measurements for the cell traces can be exported as a text file for further analysis.

1. Set the following parameters at the start:
	1. Chamber height (at the top right) – this indicates the height of the chamber in which the cells are trapped. It is used as a constraint in approximating cell volume as a 3D ellipsoid based on the major and minor axis of the cell region.
	2. μm/pixel (at the top right) – the conversion factor to calibrate distance in the images so areas and volumes can be calculated in μm2 and μm3, respectively.
	3. Filter panel (bottom left) – set minimum and maximum parameters to filter out junk regions in the mask:
		1. Area – region area in pixels.
		2. Ecc – eccentricity factor, more circular as 🡪 0
		3. SF – shape factor, more circular as 🡪 1
2. Click “Load Images” in the File I/O panel and select the data file of interest output by the FormatData GUI. For a new file, you will be asked to analyze as Timelapse or Static data. An array will automatically be generated with information for each cell region in each plane. The region mask (and sub-region mask) will be applied to each color channel and a number of different measurements are made. The data file is then saved. For a timelapse analysis, this array will be used to generate the cell time traces. (If you are loading a file into ProcessTimeSeries from a previous session, it will ask if you want to restart. BEWARE! This will erase any track curation you may have done and treat the data as if it were fresh from the FormatData GUI. If you accidentally restart, you can quickly hit Ctrl+C in the command window to prevent the \*.mat file from being overwritten.)
3. For time series data, you must next track the cells. In the “Track Cells” panel on the bottom left, set the following parameters:
	1. Max Displacement – the maximum distance a cell can travel between adjacent images before being labeled as a different cell. Higher displacement can means the algorithm can account for cells moving more, but it also increases the complexity of matching cell regions in crowds.
	2. Minimum length – minimum number of occurrences for a cell trace to be considered valid data.
	3. Frame Memory – maximum number of consecutive frames a cell region can disappear and be given the same ID when it returns. If it disappears for longer than “Frame Memory”, it will be given a new ID upon returning.
4. Click “Track Cells” to track regions from one frame to the next, and assign lineages for newly appearing buds. If an error occurs in the command window regarding “difficult combinatorics”, decrease Max Displacement and repeat tracking. The data file is saved after tracking.
5. Curate poorly segmented regions and mistakes in ID or lineage assignments using the various GUI tools or shortcut keys (shown in the bottom right panel).
	1. Slider, Prev Image, Next Image, Ctrl+left/right – use to display and move between frames in the image series
	2. Image # - displays the frame number of the current image in the right axes. Move to a specified frame by typing its number here.
	3. Delta frame – shows the difference in frame number between the right and left axes (right – left)
	4. Hide Text – toggles between displaying the cell IDs in the right axes or not.
	5. Hide Labels, Hide Mask, Overlays panel – toggles between displaying cell IDs, region mask, and color layers in the left frame. Show BF is the only one that toggles the BF layer on or off in both axes.
	6. Mask Overlay panel – choose which mask to display, “Cell” for the full cell region mask, “Nucleus” for the sub-region mask setup in the FormatData GUI, and “Cytoplasm” for the cell region not part of the sub-region.
	7. Modify Regions and Tracks panel – these controls effect changes on the cell region mask and trace information:
		1. Merge Regions (Ctrl+g or m)– smooths cell regions. Click a cell or cells in the right axes to select (region will turn red if selected). Merging on one region will morphologically close to fill in cracks or small missing chunks. Merging two or more regions will combine them into one region and give the lowest ID to the new region. Useful for over-segmented regions (often when cells have large vacuoles).
		2. Draw region – use the mouse to draw a region in the right axes where desired. Provide a unique ID.
		3. Update tracks (Ctrl+u)– use to reassign cell IDs. If no cells selected in right axes, will prompt for cell ID to change. When ID X is changed to Y, all future instances of X will be changed to Y, and any future instances of the Y, will be switched to X. (Occasionally one cell will switch back and forth between 2 IDs, repeatedly. This is due to the tracking trying to accommodate slightly oscillating regions rather than an error in the update ID function.) Multiple cells can be selected and their IDs switched simultaneously, but be sure to give each a unique ID. To assign a region to an ID that does not exist in the current file, enter “0” and one will be generated. Ctrl+w is used to swap the IDs of two highlight cells.
		4. Delete Selected Tracks (Ctrl+d) – use to delete the selected cell or multiple cell regions in the current right axes frame. (If no cells are selected, will prompt for ID.) To permanently delete all instances of an ID trace, check the box next to the Delete Selected Tracks button (text will change to “Delete ALL”). Useful for getting rid of persistent junk regions, but check future frames to make sure the ID doesn’t switch to a valid cell or bud region.
	8. Lineage Tracking panel – after tracking, lineage assignments are automatically generated. When new ID regions appears, an centroid distance minimization calculation is done to assign new regions small enough to be buds to old regions large and near enough to be mothers. The new cell ID appears in pink and a red line is drawn to the mother region. New regions too large to be buds or too far away from potential mothers appear in green and are assigned a mother ID of “NaN”. Regions existing at the first time point have a mother ID of “0”. Clicking “Calculate Lineages” at any time will recalculate all lineage assignments, including those the user has previously curated. To fix individual assignments, enter the mother and bud IDs in the text edit fields and click “Fix”. To erase a cell’s mother, enter “0” as its mother. Selecting two regions in the right axes and using Ctrl+f will automatically assign the older region as the mother and the newer region as the daughter, while erasing any previously existing mother assignments for the daughter cell.
6. Selected Cell Information panel – Get Info will display some measurements of the regions selected in the right axes. Plot Track will automatically generate a few plots of interest for the regions selected in the right axes. Can be used to determine correct IDs and lineages (e.g., if cell X has a bud at time *t*, it most likely does not have another bud at time *t*+5mins).
7. Save Changes, Ctrl+s – updates the \*.mat file to reflect user changes.
8. Generate Plots – opens the GeneratePlots GUI. Used to quickly plot selected variable information for single cells highlighted in the right axes of the ProcessTimeSeries GUI, or the mean of all cells in each frame. Can calculate rough derivatives, but be sure to specify the correct time interval. Plots can be output to a figure for saving.
9. Export Data – when cell regions are fully curated, selected variable data can be output in a \*.txt file for further analysis.