Pomegranate User Guide

Last Updated: July 06, 2020

Pomegranate Version: 1.2g

Table of Contents

Introduction

Pomegranate is a collection of macros, designed to detect, segment, and reconstruct nuclei and whole-cell 3D geometry of fission yeast from optically sectioned images.

Installation

For detailed installation instructions on downloading and installing Pomegranate using the Hauf Lab update site, see the Quick Start Guide. Alternatively, Pomegranate's macros can be directly downloaded from the Pomegranate GitHub page. Instructions in this user guide assume that you have successfully installed Pomegranate.

Included Macros

The following macros are used in the Pomegranate analysis pipeline:

The Pomegranate macro is the core macro for processing new images and is the primary macro of the Pomegranate analysis pipeline. All other macros require a completed Pomegranate analysis. This core macro either segments cells or uses an external 2D segmentation.

The Pomegranate Analysis Extension Tool extracts a ‘cell width profile’ for an existing Pomegranate analysis. The ‘cell width profile’ is a collection of radii and positions for all spheres used for extrusion.

The Pomegranate Analysis Revision Tool revises a 3D reconstruction and can perform fluorescence measurements with the revised 3D segmentation. In addition, this tool can be used to apply an existing 3D reconstruction to alternate channels.

The Pomegranate Cell Isolator Tool is used to visualize 3D reconstructions using FIJI/ImageJ’s 3D Viewer.

Additional detail on the use of each of these macros is provided in each corresponding section.

Imaging Requirements

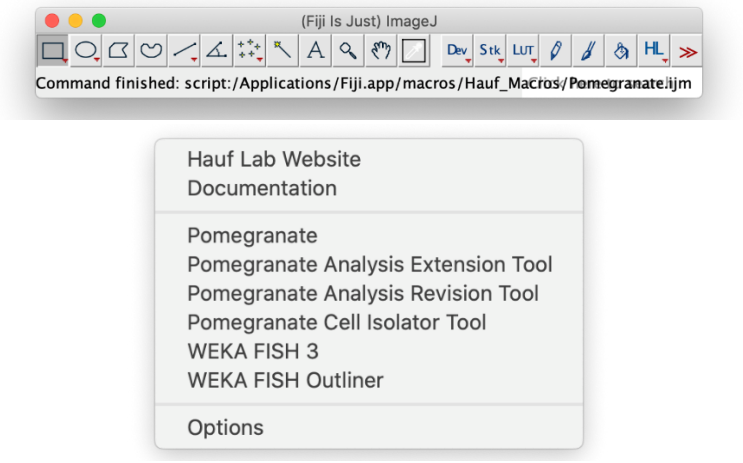
Pomegranate's nuclear segmentation and reconstruction analysis uses a Z-stack of images with nuclei marked by a fluorescent signal that is homogenous throughout the nucleus. Segmentation is optimized for fission yeast but expected to work with other cell types.

Pomegranate's whole-cell segmentation and reconstruction analysis uses a Z-stack of bright-field images. Segmentation is optimized for fission yeast, but the reconstruction is expected to work for rod-shaped cells in general.

Pomegranate's segmentation and reconstruction analyses are currently incompatible with time-lapse images.

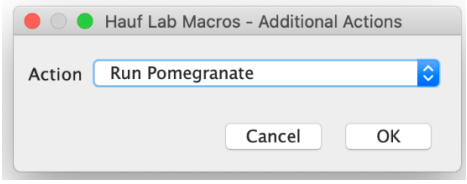
Starting Pomegranate Macros

To start Pomegranate macros from Hauf Lab Tools, locate the Pomegranate macro from the Hauf Lab Tools drop down menu. Click on Pomegranate to initialize Pomegranate.



Hauf Lab Tools Menu

Optional • To see the local copy of the source code of Pomegranate installed on your system, go to **Options ► Enable Additional Actions Prompt**



Additional Actions Prompt

If Enable Additional Actions Prompt is active, the user will be able to select between running their selected macro or viewing the local copy of the source code.

Macro Note • Macros Notes will provide brief insights into the underlying code

Sample Data and Outputs

Sample Images

Sample images, and a sample Pomegranate output are available from this Google Drive.

Sample Input – Testing Binary

Test_Binary.tif	A binary image with some pombe-shaped and other objects to check the 3D reconstruction.

Sample Input – Single Multi-Channel Image

Test_Image.tif	A four-channel Z-stack of S. pombe cells: two green fluorescence channels (Mad1-GFP), red nuclear marker (TetR-tdTomato-NLS), and brightfield

Sample Input – Multiple Single-Channel Image

BF_Test_Image.tif	brightfield
MS_Test_Image.tif	measurement signal (Mad1-GFP)
NM_Test_Image.tif	nuclear marker (TetR-tdTomato-NLS)

Note that the Sample Input for Single Multi-Channel Image, and Multiple, Single-Channel Images are identical images, simply with channels split.

Pomegranate Output File

Pomegranate provides the outputs listed below.

All auxiliary macros, such as the Pomegranate Analysis Extension Tool, the Pomegranate Analysis Revision Tool, and the Pomegranate Cell Isolator Tool, require an original Pomegranate output.

Be mindful that when renaming files or rearranging the file structure of the Pomegranate output, auxiliary macros may not function as intended when applied to these files, as they rely on a predictable naming scheme and file structure.

Sample Outputs

200529_221_BF_Test_Image.tif.zip	zip file with output from Test_Image.tif
200529_2335_Test_Binary.tif.zip	zip file with output from Test_Binary.tif

Folders / Files

…_LOG.txt	LOG file from the Pomegranate run, containing input parameters
Binaries	Images of nuclear and/or whole-cell reconstructions, prior to manual exclusion.
Results	A folder containing .csv files of the Results output. If the analysis extension tool was used, a Width Profile will also be present in this folder.
ROIs	A folder containing all ROIs as .zip files. Unfiltered vs. Filtered refers to ROIs before and after manual exclusion of objects, respectively.

Pomegranate Results CSV

The csv file ending in “Results_Full” is the primary output of Pomegranate.

Each row is a single ROI, representing a single optical section of either a nuclear or whole-cell reconstruction. The columns provide information on ROI identification, shape descriptors – and if applicable – intensities.

ROI Identifiers

Label – An ROI Label, showing the target image for intensity measurements, ROI name, and slice position in that order, delimited by colons.

Object_ID – Object ID, an Object ID generated by Pomegranate that represents a single cell. Rows (optical sections) with common Object IDs are part of the same cell and the same 3D reconstruction. Note that Object IDs from one image will all be distinct. However, occasionally, the same Object ID may be generated from a different image. Therefore, to unambiguously identify a cell, use a combination of the Image and Object_ID column.

ROI_Type – ROI Type specifies if an ROI is a midplane ROI (MID) or not (NONMID).

Data_Type – Data Type specifies if an ROI is for a Nucleus or a Whole-Cell reconstruction.

Image – The image name. In Multiple, Single-Channel input mode, this is the name of the last image provided as input.

Experiment – The experiment name, a user-defined string for annotating the whole Pomegranate run. Useful for strain information.

Misc. Information

voxelSize_X – Voxel size along the x axis.

voxelSize_Y – Voxel size along the y axis.

voxelSize_Z – Voxel size along the z axis.

voxelSize_unit – Voxel size units.

Position Descriptors

Slice – Slice number for the ROI.

X – X coordinate of the centroid of the ROI.

Y – Y coordinate of the centroid of the ROI.

XM – X coordinate of the center of mass of the ROI.

YM – Y coordinate of the center of mass of the ROI.

xpos – Ordered, comma-delimited array of X coordinates describing the ROI.

ypos – Ordered, comma-delimited array of Y coordinates describing the ROI.

Shape Descriptors

Area – ROI area in square distance units (pixels if no unit is supplied in the image properties) – in the case of the sample data, square microns.

Area_px – ROI area in pixels.

Perim. – Perimeter of the ROI in distance units (pixels if no unit is supplied in the image properties) – in the case of the sample data, microns.

Circ. – Circularity of the ROI.

Solidity – Area divided by Convex Area.

Intensity Information

Mean – Mean intensity (a.u.) within the ROI. Analogous to integrated density divided by area.

Median – Median intensity (a.u.) within the ROI.

StdDev – Standard deviation of intensity (a.u.) within the ROI.

Mode – Mode of intensity (a.u.) within the ROI.

Min – Minimum intensity (a.u.) within the ROI.

Max – Maximum intensity (a.u.) within the ROI.

Elliptical Fit Information

Major – Length of the major axis of an elliptical fit on the ROI in distance units (pixels if no unit is supplied in the image properties) – in the case of the sample data, microns. Analogous to Cell Length.

Minor – Length of the minor axis of an elliptical fit on the ROI in distance units (pixels if no unit is supplied in the image properties) – in the case of the sample data, microns. Analogous to Cell Width.

Angle – The angle between the major axis of the elliptical fit and a line parallel to the x-axis of the image.

AR – Aspect Ratio, Length of the major axis divided by the length of the minor axis.

Round – Roundness, inverse of the aspect ratio.

Feret Diameter Information

Feret – Maximum Feret diameter of the ROI in distance units (pixels if no unit is supplied in the image properties) – in the case of the sample data, microns. Analogous to Cell Length.

Min Feret. – Minimum Feret diameter of the ROI in distance units (pixels if no unit is supplied in the image properties) – in the case of the sample data, microns. Analogous to Cell Width.

FeretX – The X coordinate of the starting point for drawing the maximum Feret diameter. Using this parameter combined with FeretY, Feret, and FeretAngle will draw the Feret diameter.

FeretY – The Y coordinate of the starting point for drawing the maximum Feret diameter. Using this parameter combined with FeretX, Feret, and FeretAngle will draw the Feret diameter.

FeretAngle – The angle between the major axis of the maximum Feret diameter and a line parallel to the x-axis of the image.

Pomegranate Width Profile

The csv file ending in “Width_Profile” is a secondary output of Pomegranate, produced by the Pomegranate Analysis Extension Tool. Each row represents a single sphere from a spherical extrusion, across a whole image.

Image – The name of the input image

Object_ID – an Object ID generated by Pomegranate that represents a single cell. Rows (spherical extrusions) with common Object IDs are part of the same cell and the same 3D reconstruction. Note that Object IDs from one image will all be distinct. However, occasionally, the same Object ID may be generated from a different image. Therefore, to unambiguously identify a cell, use a combination of the Image and Object_ID column.

Type – Annotation whether a sphere is positioned along the cell body, or at the cell tip, derived from Fiji’s AnalyzeSkeleton.

Radius – Sphere Radius in pixels.

X – X position of the sphere’s center.

Y – Y position of the sphere’s center.

voxelSize_X – Voxel size along the x axis.

voxelSize_Y – Voxel size along the y axis.

voxelSize_Z – Voxel size along the z axis.

voxelSize_unit – Voxel size units.

R Analysis of Pomegranate Results

R can been used to process and visualize Pomegranate Results. Sample R code can be found in the R folder of the GitHub page. Sample R Plots can be found in the Sample Data Google Drive.

To use the R code, simply change the working directory in the setwd() line to the location of a Pomegranate output. To compile multiple Pomegranate outputs, change the working directory to the parent folder containing multiple Pomegranate outputs.

Sample R Plots

Sample R Plot 1 is a correlogram of size parameters. Note that there is no correlation for image density, as only one image is uses. Using multiple Pomegranate outputs.

Sample R Plot 2 is a comparison of Whole-Cell volumes with various reconstruction methods (‘capsule’, cylinder with hemispherical ends), where the bottom plot is Pomegranate’s reconstruction.

Sample R Plot 3 is a comparison of Nuclear volumes with various reconstruction methods (idealized ellipsoid), where the bottom plot is Pomegranate’s reconstruction.

Sample R Plot 4 is a is a comparison of Whole-Cell surface areas with various reconstruction methods (‘capsule’, cylinder with hemispherical ends), where the bottom plot is Pomegranate’s reconstruction.

Sample R Plot 5 is a is a comparison of Nuclear surface areas with various reconstruction methods (‘capsule’, cylinder with hemispherical ends), where the bottom plot is Pomegranate’s reconstruction.

Sample R Plot 6 is a scatterplot of size vs signal concentration. Note how the cells separate into two regimes, due to the mixed culture.

Pomegranate

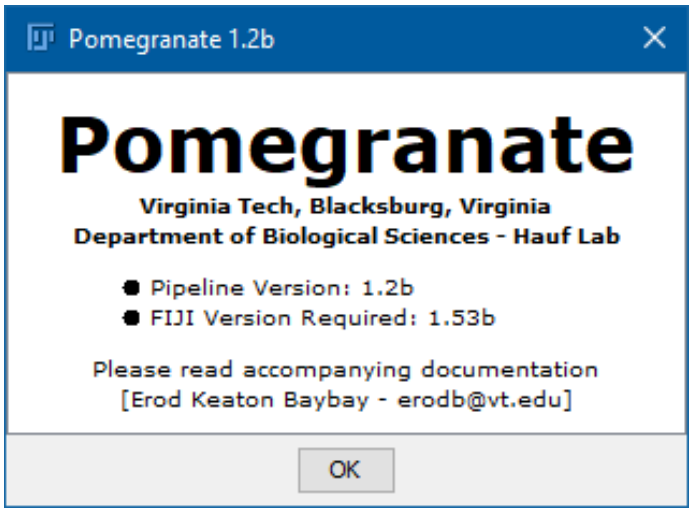
Overview

Pomegranate.ijm is the core macro of the Pomegranate pipeline and will be the starting point for all Pomegranate analyses. Pomegranate.ijm is used for detection, segmentation, and reconstruction of 3D volumes to extract shape descriptors, and – if an image or channel with a target signal is supplied – provide intensity information in 3D. All other Pomegranate tools require a complete Pomegranate output.

Pomegranate Initialization and Run Parameters

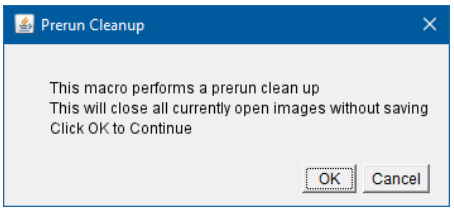
Make sure you close anything else you have been running on FIJI. Pomegranate will automatically close all your open windows. Pomegranate uses Bio-Formats (https://www.openmicroscopy.org/bio-formats/) for opening images that are not in TIF file format.

On running Pomegranate, Pomegranate's title card will appear, showing the current pipeline version and the required version of FIJI. Clicking **OK** will close the title card and resume the macro to the next step.



Pomegranate Title Card

The next window will report a warning, as Pomegranate closes all images and cleans up the following when initializing: Log, ROI Manager, Results window. Click **OK** to proceed – performing the prerun cleanup. Click **Cancel** to abort Pomegranate without performing the prerun cleanup.



Pre-Run Cleanup Warning

Macro Note • `function cleanAll(){close('*');run("Clear Results");roiManager("Reset");print("\\Clear");}`

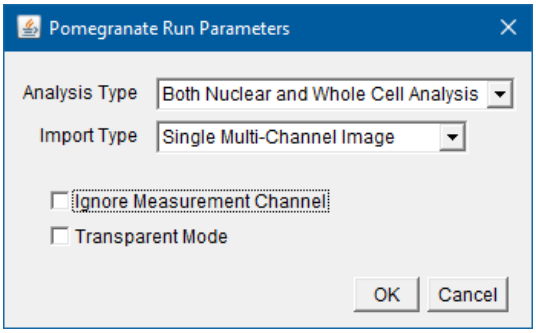
The next window represents the Run Parameters window, where you can choose between various Analysis Types: Nuclear, Whole-cell, or Both – as well as various Input Types: Single Multi-Channel Image or Multiple Single-Channel Images.

A multi-channel images has all your channels (bright-field and fluorescence) within one file. Alternatively, each channel can be input as a single-channel image. When using the test image provided (Test_Image.tif), pick “Single Multi-Channel Image” and “Both Nuclear and Whole Cell Analysis”. Separate instructions for the two options are provided below.

Enabling Ignore Measurement Channel will ignore the default requirement of a measurement channel, limiting the analysis to 2D segmentation and reconstruction only. This will result in data such as shape descriptors, but no fluorescence intensity measurements are performed.

Enabling Transparent Mode will interrupt the macro periodically to show the individual steps of the pipeline, allowing the user to inspect individual processing steps and more easily interrupt the macro when needed.

Click **OK** to proceed.



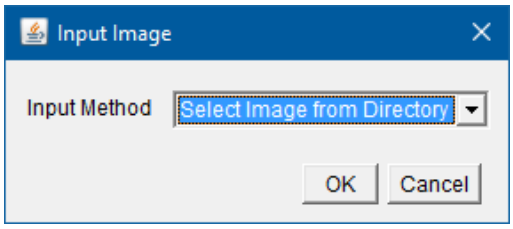
Run Parameters window.

Macro Note • Relevant Variables: `runMode, importMode, segMode, transpMode`

Input Type – Single Multi-Channel Image

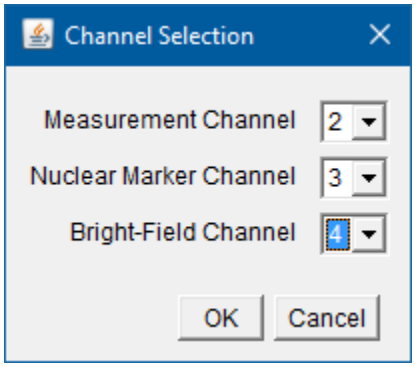
The test image is titled: Test_Image.tif found in the Multi-Channel folder.

The user will be prompted to provide the corresponding input image, either by selecting the image from their system's File Explorer, or by manually entering the path. Either option can be selected under the Input Method. Click **OK** to proceed.



Input Prompt

The next prompt is for selecting the association channels. Measurement Channel refers to the channel corresponding to the fluorescent signal that you wish to measure after 3D segmentation. Nuclear Marker Channel refers to the channel used for nuclear reconstruction. Bright-field Channel refers to the channel corresponding to the bright-field image. Note that the channel selection options will change depending on the Run Parameters chosen on the Run Parameter window. Click **OK** to proceed.



Channel Selection Prompt

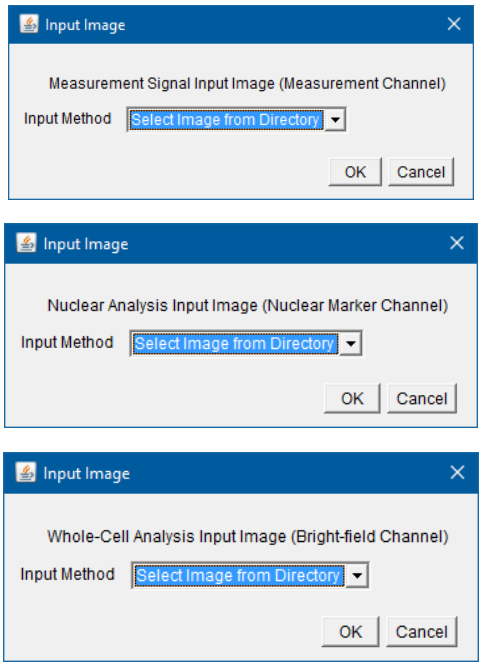
Alternative Input Type – Multiple Single-Channel Images

The test images used are: MS_Test_Image.tif, NM_Test_Image.tif, BF_Test_Image.tif, found in the Single-Channel folder.

The user will be prompted to provide the corresponding input images, either by selecting the image from their system's File Explorer, or by manually entering the path. Either option can be selected under the Input Method.

Three prompts will appear for the 'Both Nuclear and Whole-Cell Analysis' run mode. For sample images, use MS_Test_Image for the Measurement Signal Input Image, the NM_Test_Image for the Nuclear Analysis Input Image, and the BF_Test_Image for the Whole-Cell Analysis Input Image.

Click **OK** each time to proceed.

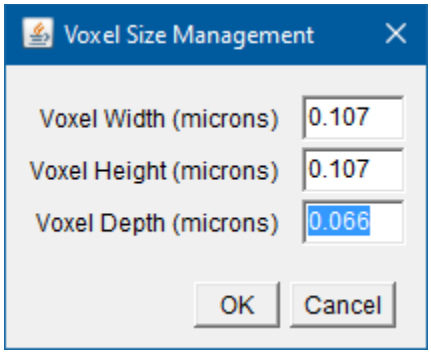


Multiple Individual Import Prompts

Additional Run Parameters

The additional run parameters are the same, regardless of whether a single image or multiple images were used as input.

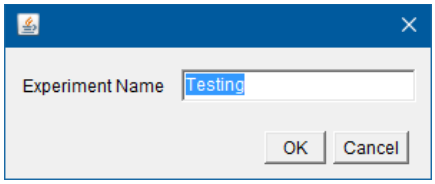
Once images have been provided, the Voxel Size Management window will appear, allowing the user to set the pixel to micron conversion for their imaging setup. This window is crucial for generating reconstructions, and volume calculations. Initial values are taken from the metadata of the image. Click **OK** to proceed.



Voxel Size Management

Macro Note • `setVoxelSize(nvx, nvy, nvz, unit);`

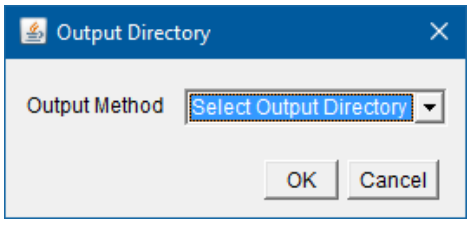
The next window is the Experiment Name prompt. Information entered here will be added in a column of the results table. This is useful for annotating strain names or imaging conditions. Being consistent in the Experiment Name labeling will make data organization of multiple Pomegranate outputs, easier. Click **OK** to proceed.



Experiment Name Prompt

Macro Note • Relevant Variables: `expName` used in `setResult("Experiment", i, expName);`

The user will be prompted to provide the output directory. This output directory will have subdirectories automatically generated. The same output directory can be used for multiple Pomegranate analyses. Click **OK** to proceed.



Output Directory Prompt

Nuclear 2D Segmentation and 3D Reconstruction

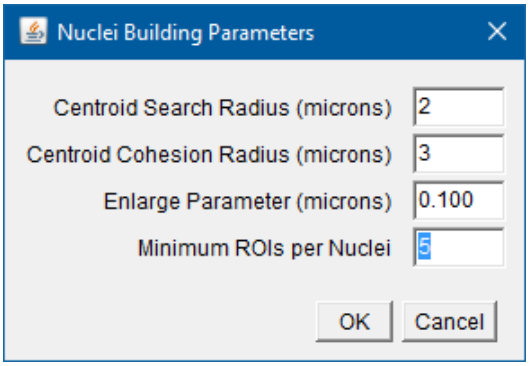
The next prompt shows the Nuclei Building Parameters, for nuclear reconstruction. The default parameters work well for the test image. Note that this step may take a while depending on the memory allocated to FIJI and your system's specifications. There will be instances where the program seems to freeze for moments – be patient as these freezes will ultimately resolve. Click **OK** to proceed.

Centroid Search Radius: This value is required for combining 2D ROIs into a 3D ROI. Centroids of 2D ROIs in adjacent sections are only considered to be part of the same nucleus, if their distance is below this value.

Centroid Cohesion Radius: This value is used to exclude nuclei that have moved during acquisition. A nucleus is discarded if any of the 2D ROI centroids has a distance of more than this value from the mean centroid.

Enlarge Parameter: Initial 2D ROIs can be extended by this value in XY. This may be required when quantifying fluorescent signals at the nuclear periphery that are not fully captured by the segmentation derived from a nucleoplasmic signal.

Minimum ROIs per Nucleus: Minimum number of 2D ROIs required to retain a 3D nuclear ROI. The value should be lower when using large Z-spacing (fewer Z sections) and higher when using smaller Z-spacing (more Z sections).



Nuclei Building Parameters

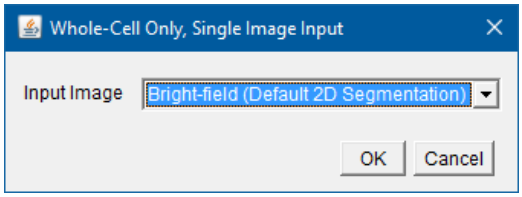
Macro Note • Pre-processing is hard coded, see the following lines, in order of appearance, preceding nuclei building parameters

```
run("Gaussian Blur...", "sigma=0.1 scaled stack"); run("Unsharp Mask...", "radius=10
mask=0.5 stack"); setAutoThreshold("Otsu dark stack"); run("Convert to Mask", "method=Otsu
background=Dark black"); run("Gaussian Blur...", "sigma=0.3 scaled stack"); run("Make
Binary", "method=Otsu background=Dark black");
```

NOTE This algorithm is not optimized for Windows systems, performance for the nuclear reconstruction may be extremely sluggish. Performance is substantially improved on Mac systems.

Whole-cell 2D Segmentation and 3D Reconstruction

The following prompt will allow you to select an input for whole-cell analysis, either a bright-field image (for use with Pomegranate's default segmentation algorithm) or a 2D binary image (from an external segmentation algorithm). Either option can be used for reconstruction.



Whole-Cell Analysis Input Type Selection Prompt

If bright-field is selected, the next prompt will show the bright-field image and suggest a focal plane (midplane).

If you disagree with the suggested mid slice, use the slider at the bottom of the bright-field image to set the slice that you consider correct. Select the slice position where most cells are in focus. If you used the slider just for checking the image, make sure it is set to your intended mid slice before you proceed.

If nuclear analysis was performed, the suggested mid slice is the mean Z position of nuclear centroids. If nuclear analysis was skipped (whole-cell only analysis) the suggested mid slice is the position where the bright-field image's standard deviation is minimized.

Note that the following image processing step is entirely hard-coded, use Transparent Mode to visualize each of the steps.

Once the image is set to the desired slice position, click **OK** to proceed.

Bright-field Focal Plane Prompt

Macro Note • Pre-processing is hard coded, see the following lines, in order by appearance, following mid-slice selection

```
run("Gaussian Blur...", "sigma=0.3 scaled"); run("Unsharp Mask...", "radius=" + getWidth() + " mask=0.90"); setAutoThreshold("Otsu dark"); setThreshold(1, 10e6); run("Convert to Mask", "method=Otsu background=Dark black"); run("Images to Stack", "name=HOLD_STACK title=HOLD use"); run("Z Project...", "projection=[Average Intensity]"); run("Auto Threshold", "method=Otsu white");
```

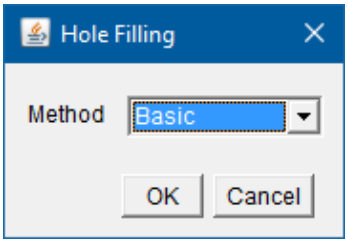
Once a binary is produced, a hole-filling prompt will appear. The user has three-options for hole-filling: None, Basic, Shape-based. Click **OK** to proceed.

None applies no hole-filling

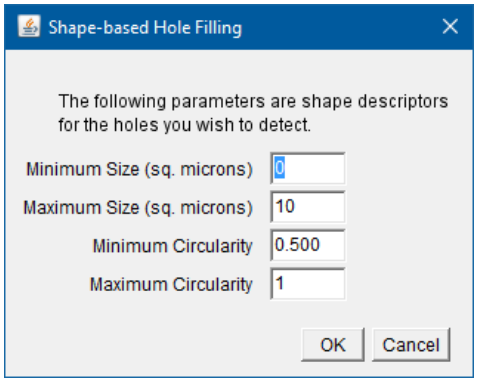
Basic applies FIJI/ImageJ's default "Fill Holes" algorithm

Shape-based searches for holes with a certain circularity, and size (Recommended)

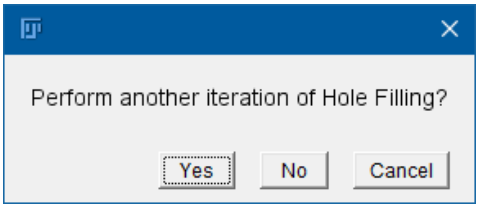
Hole-filling can be applied in multiple iterations to improve results; however, we find that one iteration is typically sufficient.



Hole-Filling Prompt

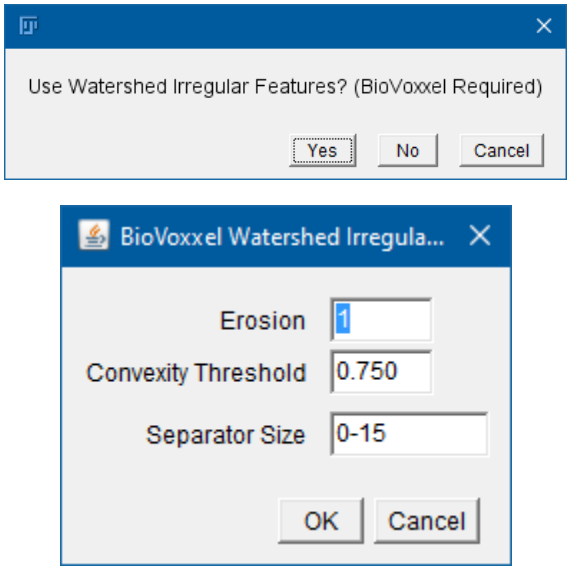


Shape-based Hole-Filling Prompt



Additional Iteration Prompt

Following hole-filling, BioVoxxel's Watershed Irregular Features can be used to further refine the binary. Adjusting the separator size will adjust the range of sizes that the algorithm will attempt to split or segment, larger sizes may lead to over-segmentation whereas smaller sizes may fail to separate joined, adjacent cells. See BioVoxxel documentation for more information on usage.

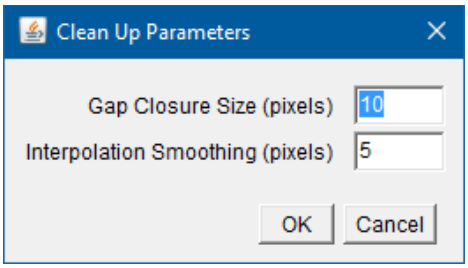


BioVoxxel Watershed Irregular Features Prompt

The next processing step is to refine and smooth edges. This is done through a method we call Gap Closure and Interpolation Smoothing.

Gap Closure fills cracks and crevices in the binary by enlarging and eroding an ROI by equal amounts, resulting in the closure of any gaps smaller than the Gap Closure Size.

Interpolation Smoothing smooths the ROI shape by using FIJI/ImageJ's "Interpolate" function, with smoothing enabled – the Interpolate function ensures that the nodes the comprise an ROI are evenly spaced at defined interval, interpolating when needed to preserve the overall ROI (Pomegranate default, 5 pixel interval). Click **OK** to proceed.



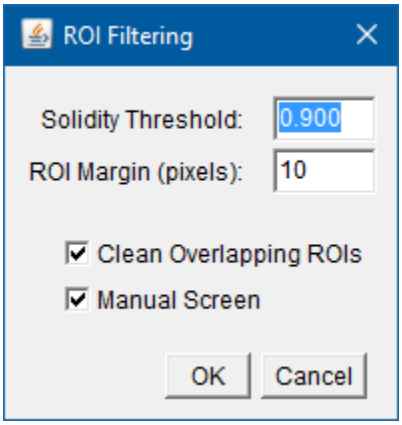
Clean Up Parameters Prompt

The result of the previous processing on the provided test image should resemble the following:

For cells at the image margin, or those that fail a solidity filter, we use a filtering scheme to exclude these cells.

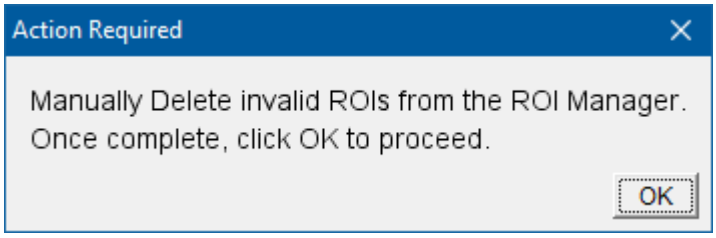
The ROI Margin is a user-defined distance from the image's edge. If an ROI overlaps with this margin at any point, it will be excluded. An ROI Margin of 0 would exclude any ROI that intersects the image edge.

The Solidity Threshold excludes any cells that fall below the Solidity Threshold (maximum: 1). A high Solidity Threshold will work for wild type cells, but a lower Solidity Threshold will be required for some mutants (e.g. bent cells). Clean Overlapping ROIs is a highly recommended step that resolves discrepancies between ROIs that overlap with each other by deleting intersecting coordinates. Manual Screen adds an optional manual filtering step prior to reconstruction. Click **OK** to proceed.

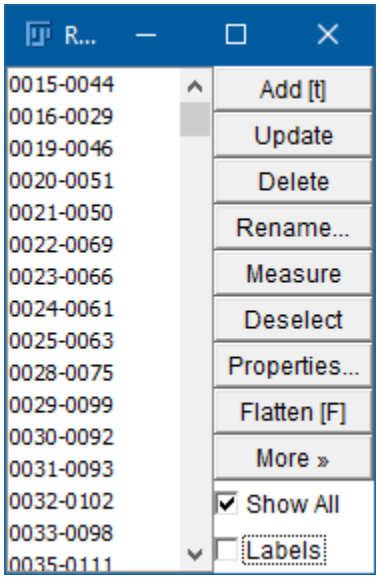


ROI Filtering Prompt

To manually delete ROIs if Manual Screen is enabled, use the ROI Manager to select erroneous ROIs, and press the Delete key, or click the Delete button to remove them. If you initially cannot see the ROIs in the ROI Manager, click on the picture. Toggle on the Labels box to show ROI labels, which can be clicked on as an alternative way to select that ROI. Clicking **OK** will proceed. Do not click OK until you are finished with manual screening.

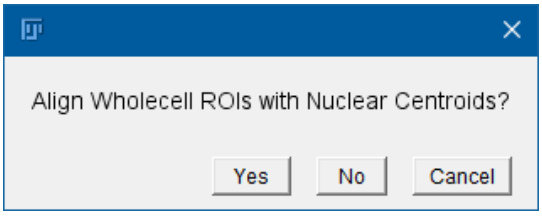


Manual Screen Prompt



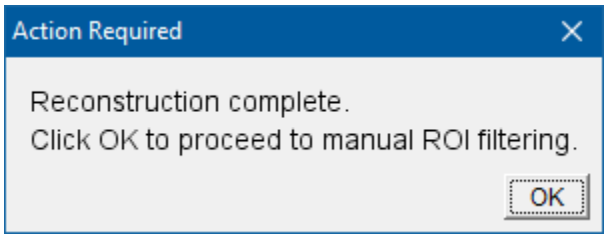
ROI Manager

If both nuclear and whole-cell analysis is chosen from Run Parameters, Whole-cell reconstructions can optionally be aligned with their corresponding nuclear centroids. By default, whole-cell reconstructions are aligned to the common mid slice set earlier. Alignments to nuclei is recommended to correct for not all cells being in exactly the same plane.



Whole-cell Nuclear ROI Alignment Prompt

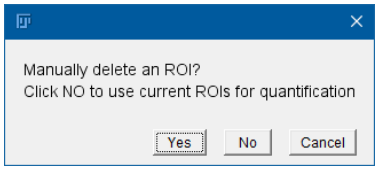
From here, reconstruction will begin, generating each whole-cell reconstruction one cell at a time. This step takes a while. Be patient. Once complete the following prompt will appear. Clicking **OK** will proceed,



Reconstruction Completion Prompt

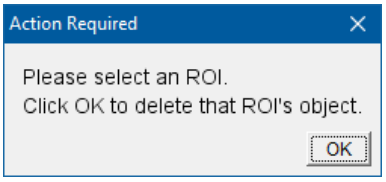
Manual Exclusion and Signal Measurement

An optional second phase of manual exclusion can be used to further refine results. Unlike during the first phase of manual exclusion, the 3D reconstruction can be assessed. Deletion is done on 'objects' (i.e. the ensemble of 2D ROIs that form one 3D ROI). Do not use the Delete key or the Delete button on the ROI Manager, in this instance, but follow the instructions below.

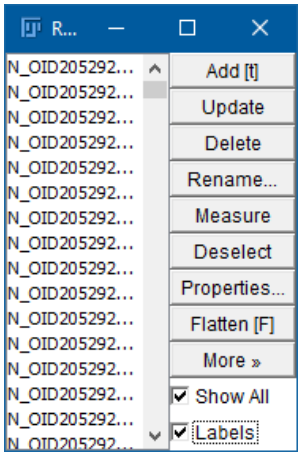


Manual Exclusion Prompt

If **Yes** is selected in the previous prompt, select an ROI by clicking on its label, then click OK to delete it. Do not use the ROI Manager to delete. You can use the ROI Manager to toggle Labels, though.

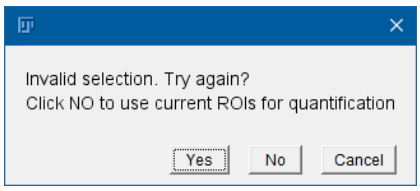


Manual Exclusion Selection Prompt



ROI Manager

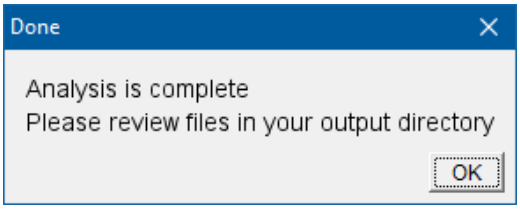
If no ROI is selected, when OK is clicked, the following prompt will appear:



Invalid Selection Prompt

Manual exclusion will happen for multiple iterations until No is clicked. Once No is clicked, ROIs are submitted for measurement, and the Measurement Signal Input Image / Measurement Channel will be analyzed with the reconstructions.

Note that if Ignore Measurement Channel was enabled in the Run Parameters, the outputs will instead be primarily shape descriptors. The following prompt will appear once the analysis is complete.



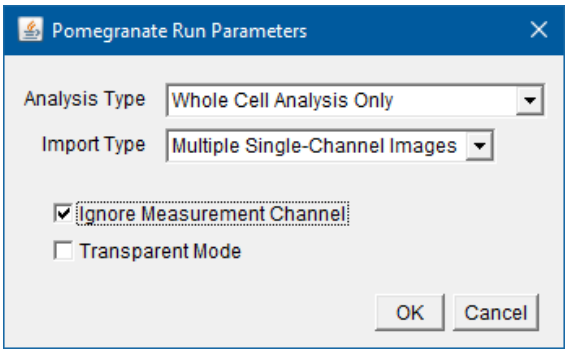
Completion prompt

NOTE This algorithm is not optimized for Windows systems, performance for the results extraction may be extremely sluggish. Performance is substantially improved on Mac systems.

Using External Binaries as Inputs for Pomegranate Reconstruction

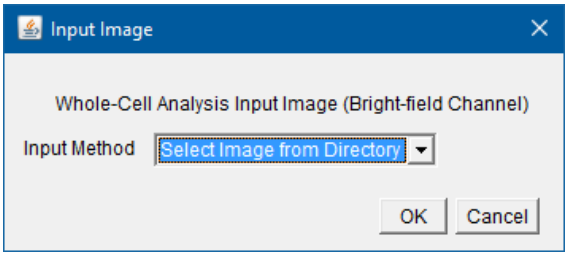
The sample image used in this section is Test_Binary.tif

To apply Pomegranate's reconstruction algorithm to an existing segmented binary image of fission yeast (or any binary image), use the following run parameters as the beginning of the Pomegranate analysis. Click **OK** to proceed.



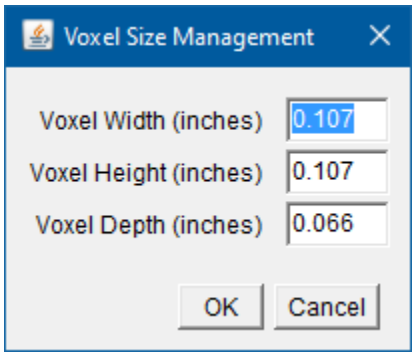
Run Parameters Prompt

The following prompt will appear, where the user may choose their preferred input method. Note that while the prompt may request a bright-field image, a binary can be accepted. Click **OK** to proceed.



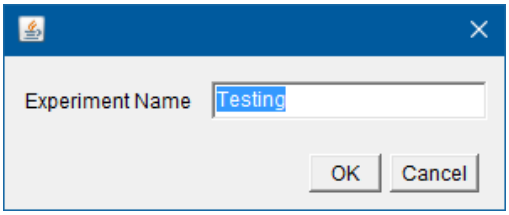
Input Prompt

The next prompt will request a voxel size for the reconstruction. This window is crucial for generating reconstructions, and volume calculations. Initial values are taken from the metadata of the image. Click **OK** to proceed.



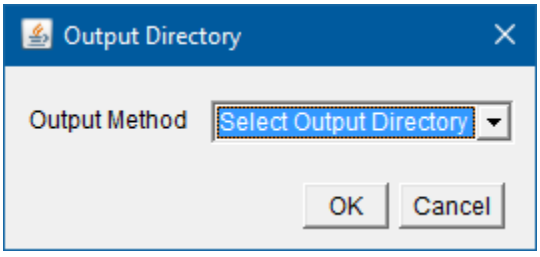
Voxel Size Management Input Prompt

As with normal Pomegranate runs, an Experiment name can be set in the next prompt. This information will be added as a column in the output. Click **OK** to proceed.



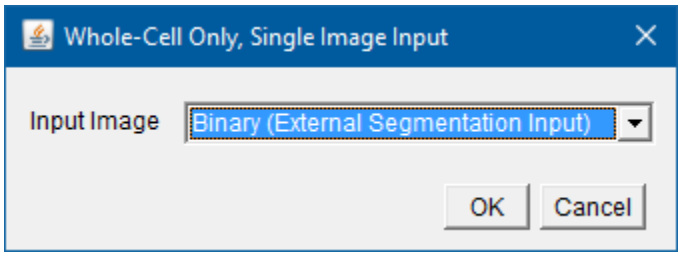
Experiment Name Prompt

The user will be prompted to provide the corresponding output directory. The same output directory can be used for multiple Pomegranate analyses. Click **OK** to proceed.



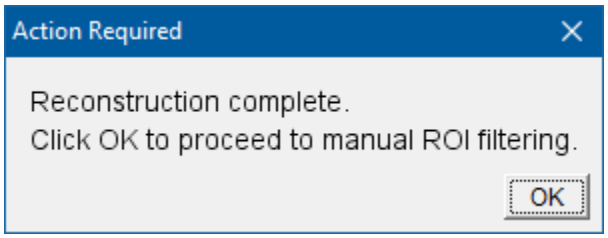
Output Prompt

Once an output directory is provided, the user will be prompted to declare the input image type. Ensure that this is set to Binary (External Segmentation Input).



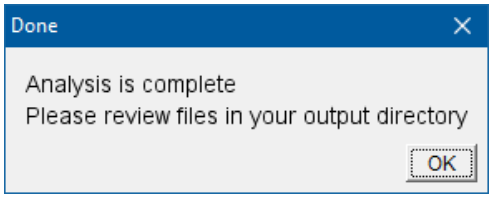
Whole-Cell Analysis Input Type Selection Prompt

From here, reconstruction will begin, generating each whole-cell reconstruction one cell at a time. This step takes a while. Be patient. Once complete the following prompt will appear. Clicking **OK** will proceed,



Reconstruction Completion Prompt

Continue as a normal Pomegranate analysis through manual exclusion and results compilation. Once the following prompt is shown, the analysis is complete.



Completion Prompt

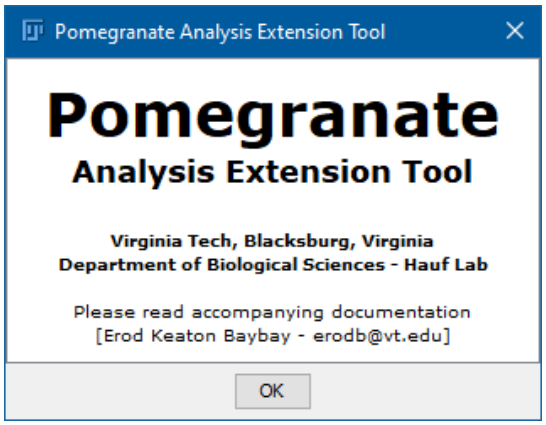
Pomegranate Analysis Extension Tool

Overview

Pomegranate_Analysis_Extension_Tool.ijm is a tool that expands upon an existing Pomegranate analysis, exporting cell width profiles for existing reconstructions. A cell width profile will give information on the individual spheres that comprise the extrusion-based whole-cell reconstruction. This tool requires a complete Pomegranate analysis.

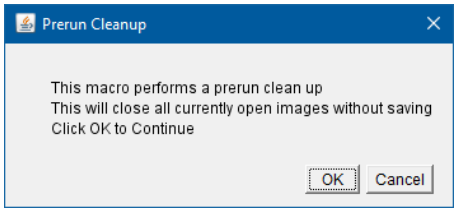
Initialization and Usage

On running Pomegranate Analysis Extension Tool, a title card will appear. Clicking **OK** will close the title card and resume the macro to the next step.



Pomegranate Analysis Extension Tool Title Card

The next prompt will warn of a prerun cleanup. Click **OK** to proceed – performing the prerun cleanup. Click **Cancel** to abort Pomegranate without performing the prerun cleanup.



Pre-Run Cleanup Warning

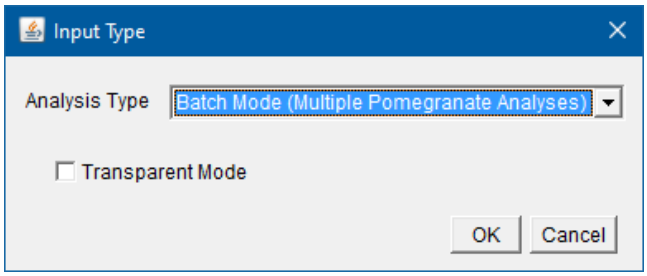
Macro Note • `function cleanAll(){close('*');run("Clear Results");roiManager("Reset");print("\\Clear");}`

The next prompt will allow the user to select an input type. If multiple Pomegranate Analyses are stored in the same directory, then Batch Mode can be employed to analyze all inputs at once.

Alternatively the macro can be run in Single Mode to apply the analysis extension to a single analysis.

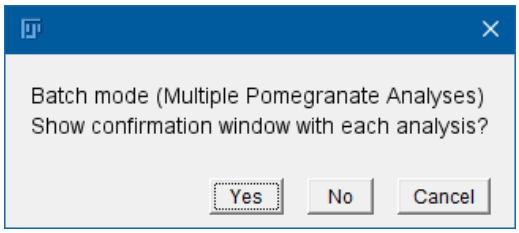
Enabling Transparent Mode shows all processing steps applied to the image. Otherwise, with Transparent Mode disabled, all processing steps will be hidden to speed up the analysis.

Here we will use Single Mode for the sample output dataset. Click **OK** to proceed.



Analysis Type Prompt

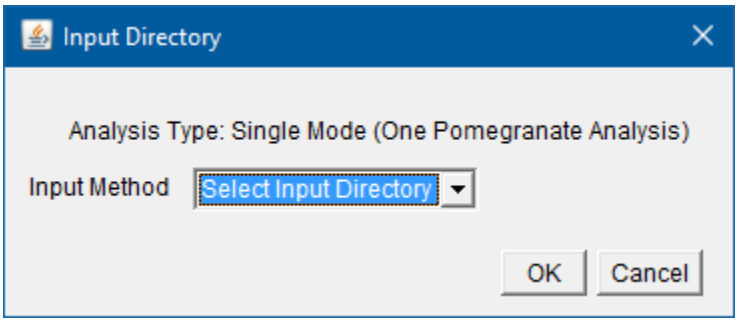
If Batch mode is used, the following prompt will appear, allowing the analysis to be interrupted in between each Pomegranate output extension task. Otherwise the macro will perform the analysis extension without halting. This window will not appear for Single Mode.



Confirmation Window Prompt

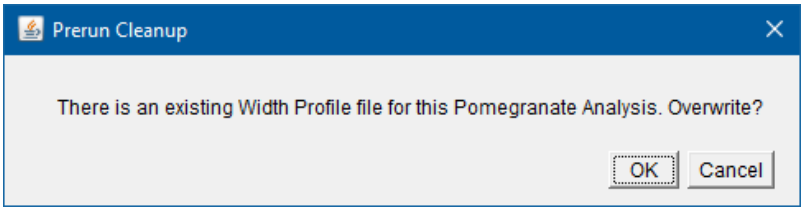
The next prompt will request an input directory. In Single Mode, a single Pomegranate output file is selected. In Batch Mode, the directory containing multiple Pomegranate outputs is selected. The Analysis Type will be displayed at the top of the window.

Click **OK** to proceed.



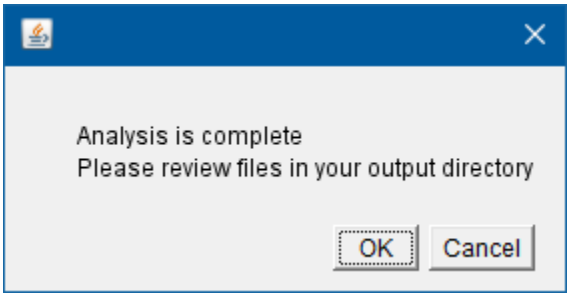
Input Prompt

The analysis extension will carry on from here, and a Width profile will be saved to the Results folder of the selected Pomegranate output. If there is an existing Width Profile, the following prompt will appear, offering the option to overwrite the existing Width Profile.



Overwrite Width Profile Prompt

If the analysis detects no Width Profile, the following will completion prompt will be provided.



Completion Prompt

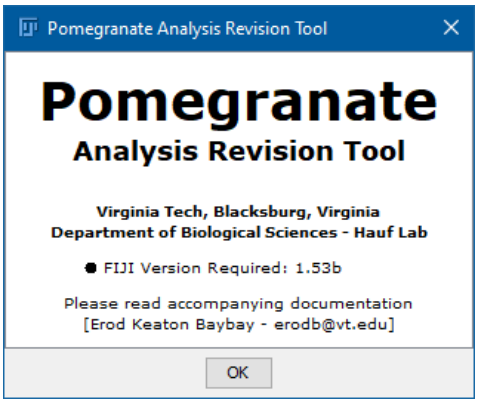
Pomegranate Analysis Revision Tool

Overview

Pomegranate_Analysis_Revision_Tool.ijm is used for reevaluating a Pomegranate reconstruction. It's primary uses are to reperform reconstruction calculations under altered voxel size parameters, or to apply the same reconstructions to different target signal channels. This tool can also be used to redo the final manual exclusion step in a Pomegranate analysis, able to recover reconstructions prior to manual exclusion. This tool completely bypasses any early segmentation or preprocessing, focusing solely on reevaluating reconstruction and signal measurement. This tool requires a complete Pomegranate analysis.

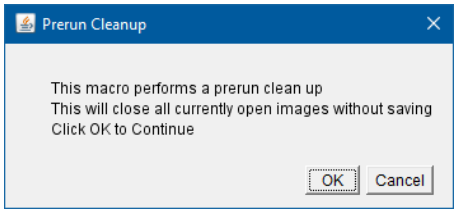
Initialization and Usage

On running Pomegranate Analysis Revision Tool, a title card will appear, showing the required version of FIJI. Clicking **OK** will close the title card and resume the macro to the next step.



Pomegranate Analysis Revision Tool

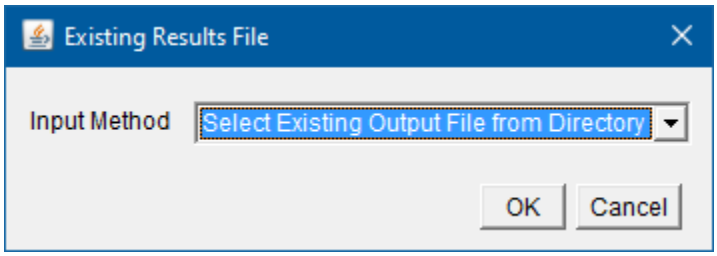
The next prompt will warn of a prerun cleanup. Click **OK** to proceed – performing the prerun cleanup. Click **Cancel** to abort Pomegranate without performing the prerun cleanup.



Pre-Run Cleanup Warning

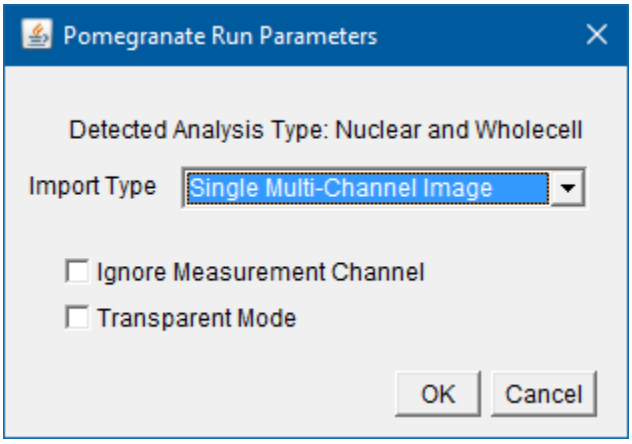
Macro Note • `function cleanAll(){close('*');run("Clear Results");roiManager("Reset");print("\\Clear");}`

Following the prerun clean-up, the user will be prompted to select an existing Pomegranate output folder.



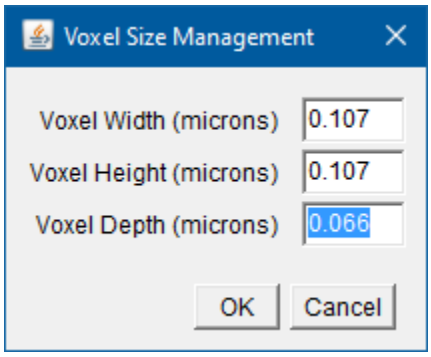
Input Prompt

The reanalysis macro will automatically detect the input type, based on the ROIs stored in the ROI folder. Import Type, Ignore Measurement Channel, and Transparent Mode are identical in functionality to the Pomegranate Run Parameters of the core Pomegranate macro. See the Pomegranate Run Parameters section of the core Pomegranate macro for details on these options.



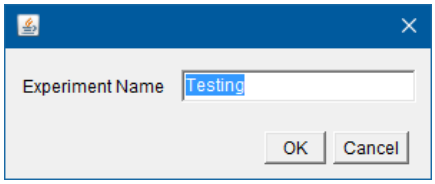
Pomegranate Run Parameters

The following prompts are the same as the Additional Run Parameters present in the Pomegranate core macro. See the Additional Run Parameters section of the core Pomegranate macro for details on the following prompts.



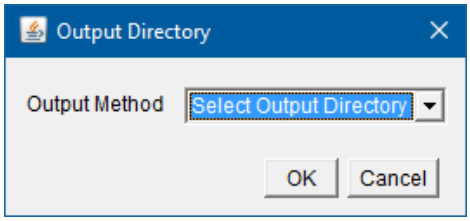
Voxel Size Management

Macro Note • `setVoxelSize(nvx, nvy, nvz, unit);`



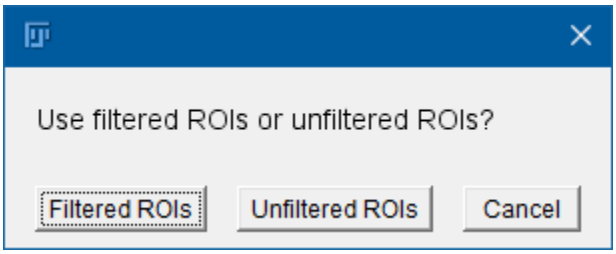
Experiment Name Prompt

Macro Note • Relevant Variables: `expName` used in `setResult("Experiment", i, expName);`



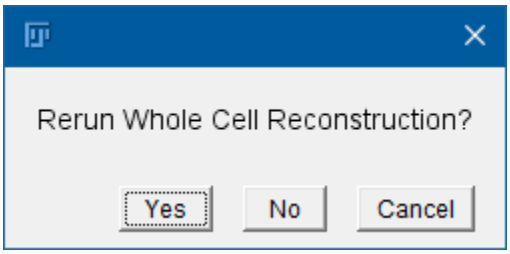
Output Directory Prompt

The next prompt will ask the user if they want to use filtered ROIs or unfiltered ROIs. The filtering here refers to the final manual exclusion step in Pomegranate's core analysis. Choosing unfiltered ROIs will undo the manual exclusion, allowing the user to redo the manual exclusion here.



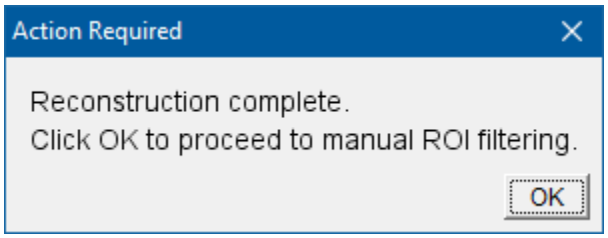
Filtered ROI Prompt

The next prompt will ask the user if they want to rerun the Whole Cell Reconstruction, recalculating the reconstruction from scratch. Rerunning the whole cell reconstruction is necessary for accurate reconstruction under altered voxel sizes.

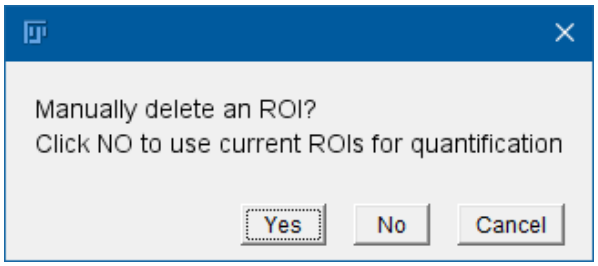


Rerun Reconstruction Prompt

The following prompts are identical to the prompts described in the Manual Exclusion and Signal Measurement section of the core Pomegranate analysis macro.



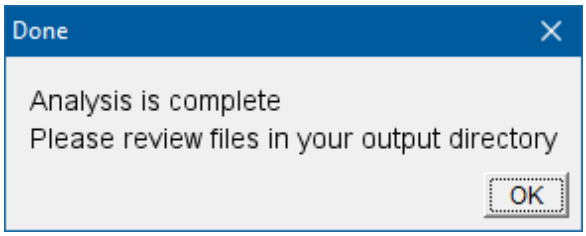
Reconstruction Completion Prompt



Manual Exclusion Prompt

Manual exclusion will happen for multiple iterations until No is clicked. Once No is clicked, ROIs are submitted for measurement, and the Measurement Signal Input Image / Measurement Channel will be analyzed with the reconstructions.

Note that if Ignore Measurement Channel was enabled in the Run Parameters, the outputs will instead be primarily shape descriptors. The following prompt will appear once the analysis is complete.



Completion prompt

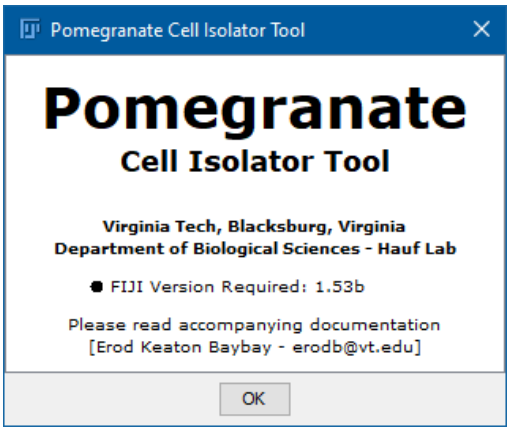
Pomegranate Cell Isolator Tool

Overview

Pomegranate_Cell_Isolator_Tool.ijm is used to visualize individual cells using Fiji's built-in 3D Viewer, with consistent parameters. It is also used to quickly extract cell shape descriptors of individual cells, as well as visualize overlays idealized cross sections (cross section of a straight rod-shaped capsule) on the binary cross section of the cell. This ideal reconstruction comparison is used to see sources of discrepancy between idealized approaches at calculating cell volume, and Pomegranate's method. This tool requires a complete Pomegranate analysis.

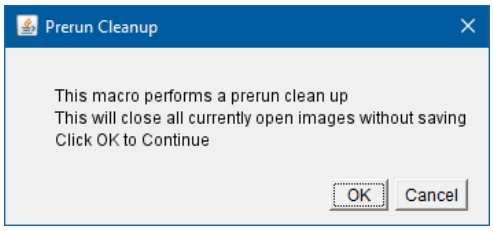
Initialization and Usage

On running Pomegranate Cell Isolator Tool, a title card will appear, showing the required version of FIJI. Clicking **OK** will close the title card and resume the macro to the next step.



Pomegranate Cell Isolator Title Card

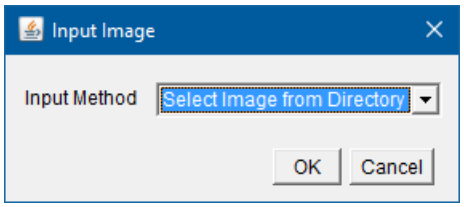
The next prompt will warn of a prerun cleanup. Click **OK** to proceed – performing the prerun cleanup. Click **Cancel** to abort Pomegranate without performing the prerun cleanup.



Pre-Run Cleanup Warning

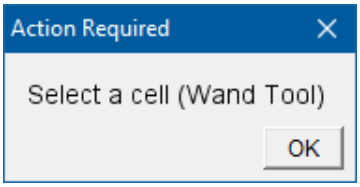
Macro Note • `function cleanAll(){close('*');run("Clear Results");roiManager("Reset");print("\\Clear");}`

Next is to select an input image. This is different from the input image in Pomegranate base analysis. The input image required for this tool is the Whole-Cell RGB image in the Binaries folder of the Pomegranate output.

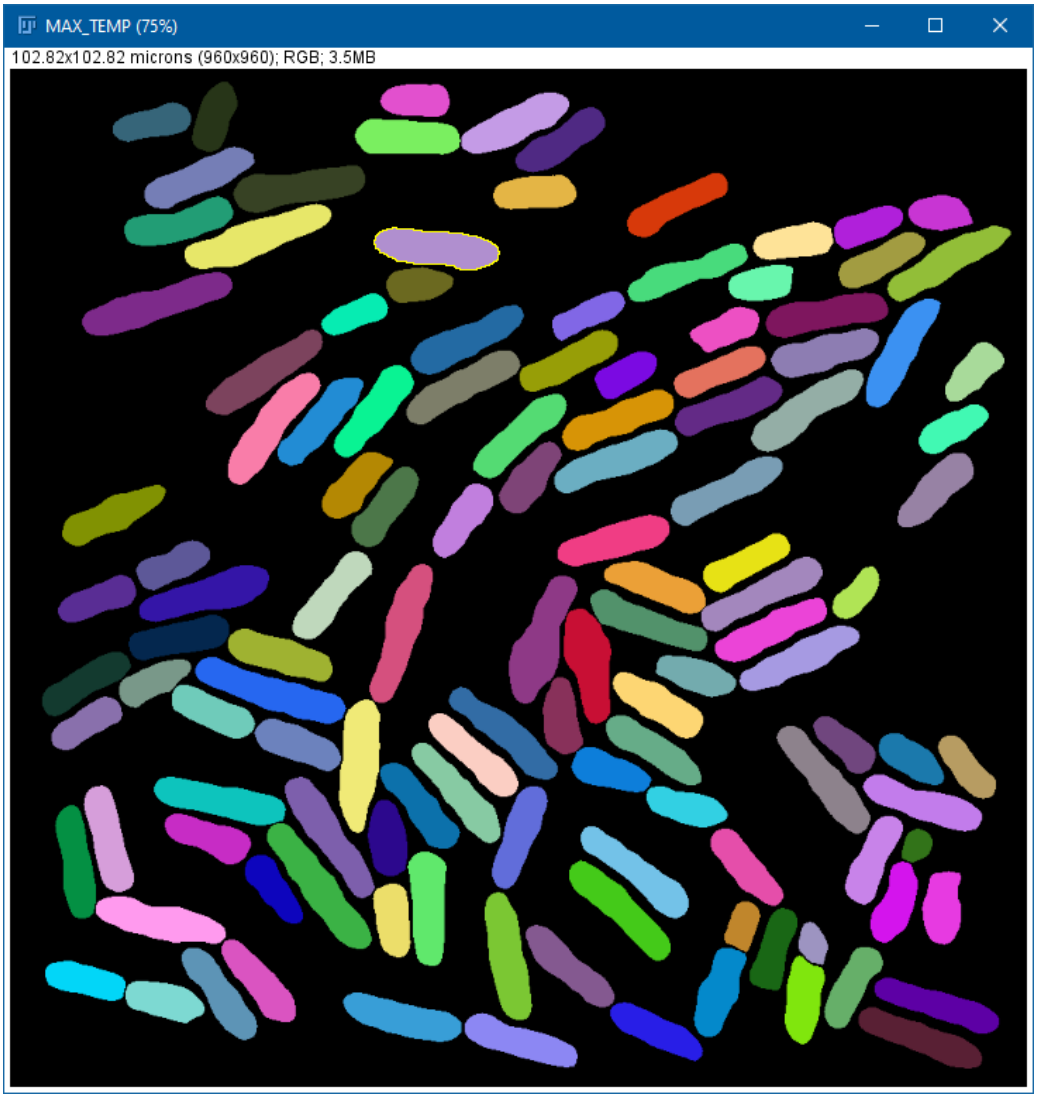


Input Prompt

If an image successfully opens, the following prompt will appear. The wand tool will automatically be selected, simply click the desired cell for isolation. Do not change active images, ensure that you are selecting the cell from the MAX_TEMP window.

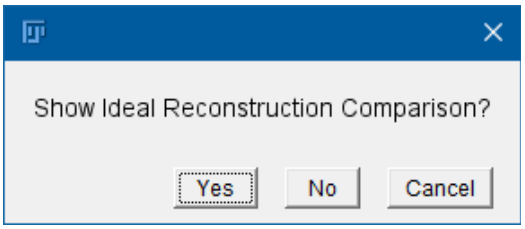


Wand Prompt

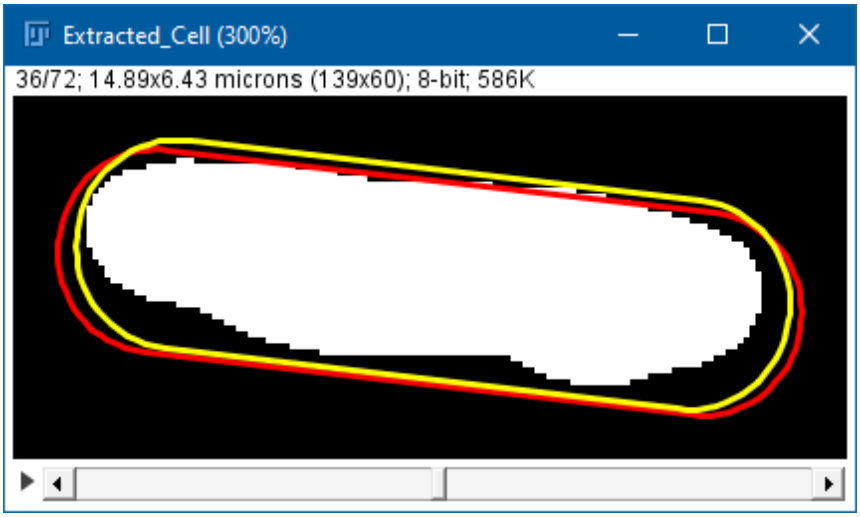


MAX_TEMP Selection Window

A prompt will appear, offering to show cross-sections of idealized volumes overlaid upon the selected cell. Clicking **No** will skip this step.



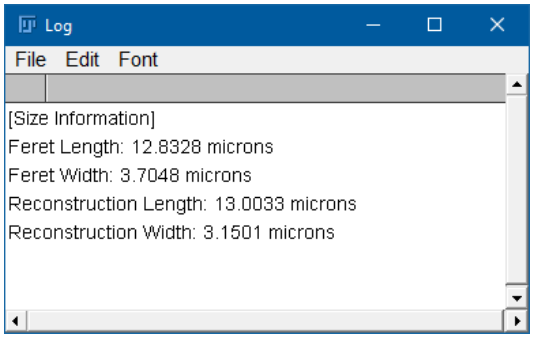
Ideal Reconstruction Comparison Prompt



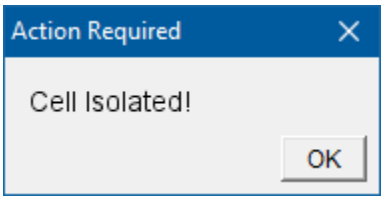
Ideal Reconstruction Comparison

The final step of the cell-isolator tool will show the reconstruction in the ImageJ 3D viewer. Additionally, size information will pe provided in the Log file, and a prompt showing the completion of the macro will appear. Note that no files will be automatically saved or output.

ImageJ 3D Viewer



Cell Isolator Log



Completion Prompt