

OrganoSeg2 ReadMe

Table of Contents

Summary	4
Installation	6
Loading New Images	7
Image Preparation	8
<i>Crop Image</i>	8
<i>Brightness</i>	8
<i>Resize</i>	9
Image Grouping	10
Segmentation	11
<i>Corrections</i>	12
<i>Out-of-Focus Correction</i>	12
<i>DIC Correction</i>	12
<i>Watershed Split</i>	13
<i>Edge Correction</i>	13
<i>Parameters</i>	14
<i>Intensity Threshold</i>	14
<i>Window Size</i>	14
<i>Size Threshold</i>	14
<i>Edge Intensity</i>	14
<i>Contaminant Intensity</i>	15
<i>Minimum Circularity</i>	15
<i>Additional Parameters</i>	16
GUI Layout	17
<i>Panel Resizing</i>	17
Spheroid Editing Toolbar	20
<i>Remove</i>	20
<i>Combine</i>	20
<i>Individual Split</i>	20
<i>Individual Edge Correct</i>	21
<i>Change Classification</i>	22
<i>Undo</i>	23
Metric Selection Tool	24
Pixel Calibration Tool	25

Save Images	26
<i>Current Images.....</i>	26
<i>Image Set.....</i>	26
Export	27
Loading Segmented Images	29
Tracking Analysis	30
Fluorescence Analysis	36
<i>Fluorescence Analysis of Tracked Organoids</i>	39
Keyboard Commands	41
Currently Known Bugs.....	42

Summary

Segmentation Analysis

1. **Open new images** using File → Open → New Image Set or Ctrl+o (or Command+o if using MacOS).
2. **Perform in-app image adjustments** as needed, including brightness/contrast adjustments, cropping, rotation, or image resizing, which appear in the Raw Grayscale Image panel and Preferences menu bar.
3. (Optional) **Group images** based on filename contents using the Grouping Options tab in the bottom panel. Enter a regex expression with variable names, and confirm grouping classifications using the Groups tab on the left panel. Using the Grouping Options tab to establish how images will be grouped for export or batch tracking.
4. **Set segmentation parameters** in the Segment Options tab and Preferences → Additional Parameters, Edge Correction.
5. **Segment images** using the buttons on the left. Optionally set the number of workers for parallelization.
6. **Perform post-segmentation tuning** using the toolbar below the image displays.
7. **Select metrics and pixel calibration** using Preferences → Metrics, Calibration.
8. **Save segmentation images** using File → Save.
9. **Export segmentation data** to an Excel sheet, or GUI data to a .mat file to be opened/modified later using File → Open → Previously Segmented Images.

Tracking Analysis

1. With segmented images in the workspace, go to Additional Analysis → Tracking. If applied grouping (step 3), use Track in Batches. Otherwise, use the Track Single Batch option.
2. **Select images** to be included in the analysis and press begin tracking. If tracking in batches, selected images which do not meet the specified grouping criteria will not be included. If tracking a single batch, select images in chronological order.
3. (Optional) If using grouped images, go to the Sorting tab and select a variable to **sort each batch's images** with. Then, click the variable values in the desired order.
4. **Register** images with the Registration tab. Confirm images are correctly aligned, and then click Done Orienting.
5. Specify a maximum distance, minimum/maximum growth between matched organoids, and then press **Track**.

6. If desired, **manually verify organoid traces** and classify poor or exemplary traces using “x” and “c” on the keyboard. Use options at the bottom of the screen to adjust tracking as needed.
7. **Export** segmentation data to an Excel sheet, or GUI data to a .mat file to be opened/modified later using the Load Saved File button from Additional Analysis → Tracking on the main scene. For Excel export, indicate which organoid traces you would like to export.

Fluorescence Analysis

1. Open the fluorescence analysis scene.
 - a. From the main segmentation scene, go to Additional Analysis → Fluorescence. **Select brightfield images** to be included in the analysis and press Begin Fluorescence.
 - b. If performing fluorescence analysis on tracked images, click on the Fluorescence button in the menu bar of the tracking scene. This will open fluorescence analysis on the currently displayed image batch.
2. In the fluorescence scene, go to File → Open Fluorescence Images or Ctrl+o (or Command+o if using MacOS) and **open fluorescence images** that correspond to your selected brightfield images. Click Done Opening Images when finished.
3. **Match fluorescent images to brightfield images** using either the Images Are in Order checkbox, the Image Assignment Tab, or manual assignment using the Assign Image button. Once finished, click Done Assigning.
4. Select a representative pixel intensity and **analyze images** using the Analyze Current or Analyze All buttons.
5. **Visualize results** using the Data Display tab, and change the fluorescent cutoff as desired by clicking on the histogram or changing the value in the edit field.
 - a. If analyzing tracked organoids, use the See Montage button to see individual organoid fluorescence over time.
6. **Save fluorescence overlay images** using File → Save.
7. **Export** segmentation data to an Excel sheet, or GUI data to a .mat file to be opened/modified later using the Load Saved File button from Additional Analysis → Fluorescence on the main scene. For Excel export of tracked organoids, indicate which organoid traces you would like to export.

Chapter 1: Installation

Installing and running the standalone (.exe/.app) implementation

1. Download and run the appropriate installer
 - a. To install the MATLAB runtime environment automatically, use the OrganoSeg2Installer_web.exe (Windows) or OrganoSeg2Installer_web.app (MacOS)
 - b. If you already have MATLAB installed, use the OrganoSeg2.exe (Windows) or OrganoSeg2.app (MacOS). Please ensure that the version of MATLAB installed is version 2024b or more recent. OrganoSeg2 will run on earlier versions, but certain functions (e.g., image registration for organoid tracking) will not function optimally.
2. Follow installation instructions. Check “Create Desktop Icon”.
3. Double-click the installed OrganoSeg2 icon.

Installing and running the MATLAB implementation

1. Place the following files into an active MATLAB directory. This can be done by extracting the zipped files into the directory or by dragging and dropping them. Please ensure that the version of MATLAB installed is version 2024b or more recent. OrganoSeg2 will run on earlier versions, but certain functions (e.g., image registration for organoid tracking) will not function optimally.

Files needed:

- adaptivethreshold.m
- brightnessSelection.mlapp
- fluorescenceAnalysisScene.mlapp
- imoverlay.m
- metricSelectionUpdate.mlapp
- nextname.m
- nonRigidRegisterFunction.m
- OrganoSeg2.mlapp
- PoolWaitbar.m
- radialpoly.m
- rigidRegisterFunction.m
- segmentFunction.m
- trackingAnalysisScene.mlapp
- Zernike_main.m
- Zernikmoment.m

MATLAB Toolboxes:

- Image Processing Toolbox
- Statistics and Machine Learning Toolbox
- Parallel Computing Toolbox (Optional)

2. Ensure the files are all on the MATLAB path.

3. Either load the OrganoSeg2.mlapp script and press "Run", or type OrganoSeg2 into the MATLAB command window.

Chapter 2: Loading New Images

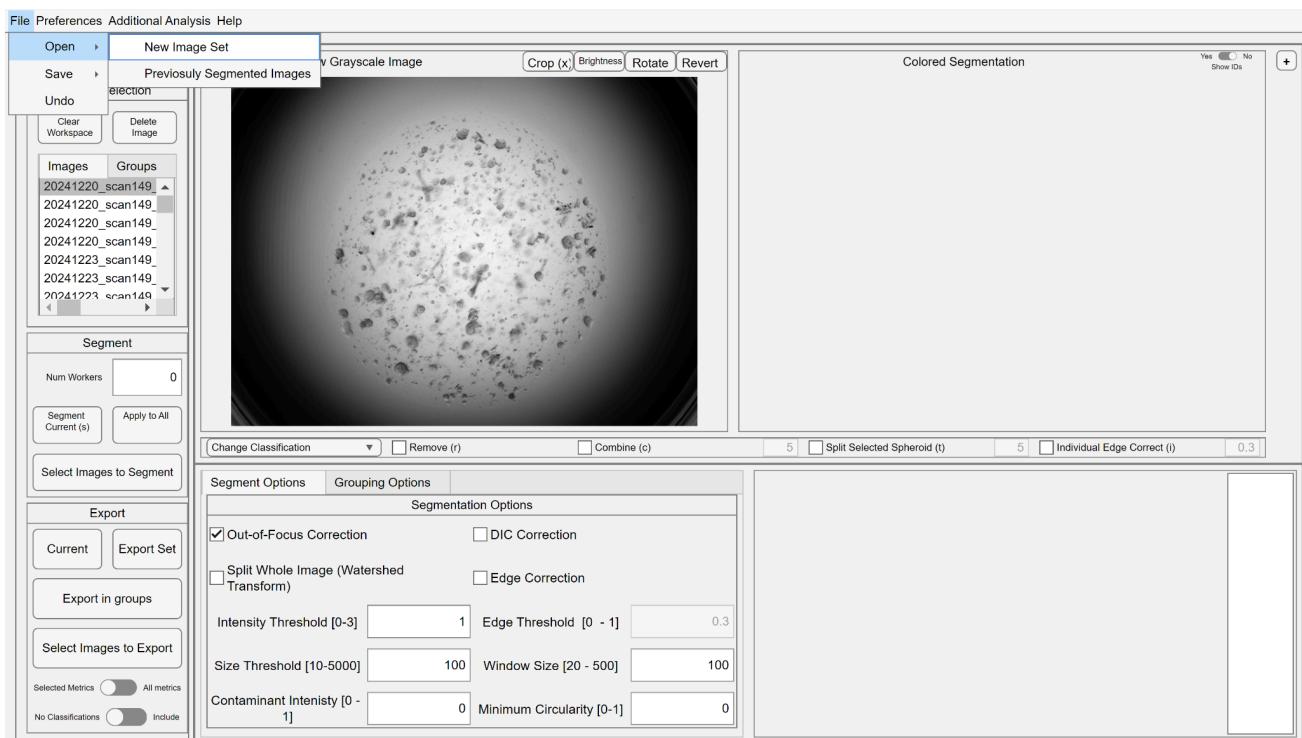
File selection (File → Open → New Image Set or Ctrl+o):

Click the image you wish to load. To load multiple images concurrently, hold Shift and select the image that is at the end of the range you would like to upload. To load multiple non-sequential images, hold Control and select the additional images. Click open to finalize the upload of the highlighted images. To open images from separate directories, this process must be repeated for each directory.

Images will appear in the “Image Selection” list. Scrolling through this list will show the selected image in the “Raw Grayscale Image” panel.

Once images have been uploaded, the same process can be repeated to add more images to the workspace without removing data from the previously uploaded images.

If you are done working with images, select “Clear Workspace” in the main scene to remove images and their associated data. You will be prompted to confirm whether or not you want to clear the work space.



Chapter 3: Image Preparation

Crop Image

Select the “Crop” button (or press “x”) to crop the image in-app. Cropping reduces unwanted segmentations by only segmenting the desired region of interest, and improves runtime given the smaller image size. Cropping style is either rectangular, circular, or freehand. Crop style is changed in Preferences → Cropping. Cropping changes are undone by pressing “z.”.

Rectangular: Select two opposite corners of a rectangle.

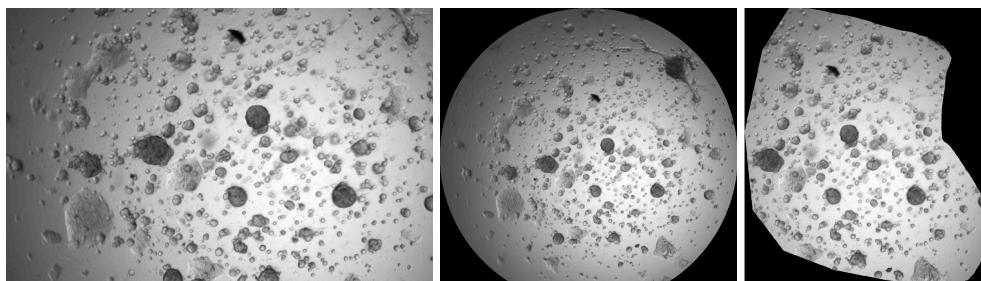
Circular: Select the center and then any point along the perimeter of the circle.

Freehand: Trace the region of interest (may cause program to lag).

The image will fill into a rectangular shape with black space for circular and freehand crop.

Press ‘esc’ at any point to exit the cropping process.

For Rectangular and Circular cropping, once you perform a crop, you will have the option to “Use Last Crop Dimensions.” If selected, you will only need to select the first corner or the center, respectively, and the crop dimensions will be automatically specified.



Brightness

Preference (Preferences → Brightness): Opens up a new scene which allows the user to test different combinations of contrast adjustments on the image and see the effect on segmentation. Possible contrast adjustments include the following MATLAB functions: imlocalbrighten, imflatfield, imlocalcontrast, imadjust, locallapfilt, imreducehaze, and histeq. See MATLAB documentation for parameter information.

Once done testing adjustments, return to the main scene and press the “Brightness” button at the top of the “Raw Grayscale Image” panel, and then apply the changes one at a time. This may be done to only the selected image or to all images. Applying brightness changes will reset segmentation data. Brightness changes are undone by pressing “z.” If pressing “z” to undo does not work, click anywhere on the app that is not a button or other UI component to reengage the keystroke functions.

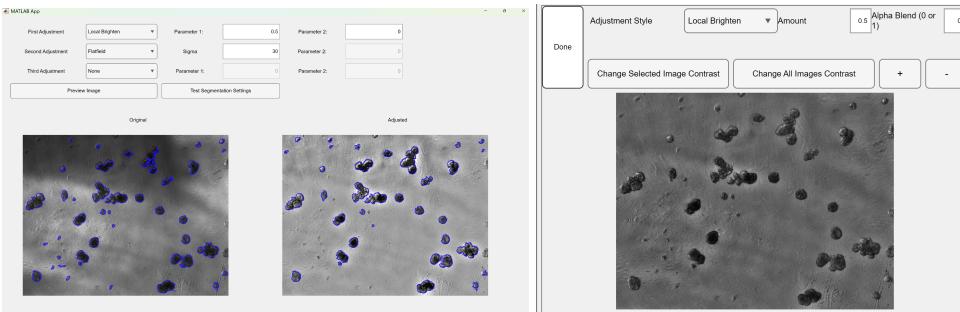
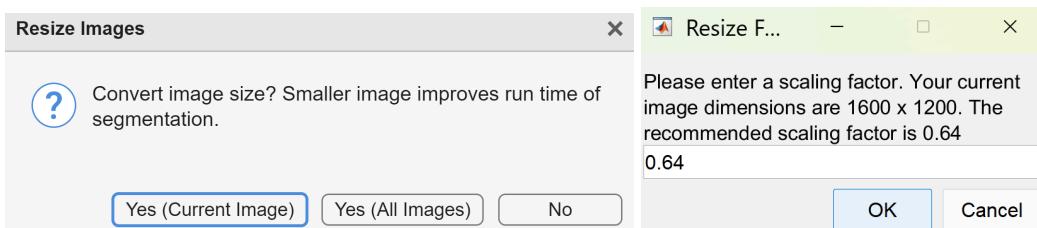


Image resize (Preferences → Resize Images)

Image resizing quickens the segmentation by down-sampling large images for adaptive thresholding and then enlarging the segmented boundaries for metric export. This may reduce segmentation quality.

Users are first prompted to resize only the current image, resize all images, or cancel. If either “Yes” option is selected, they are prompted to enter the resize factor, which is set to the default resize factor in OrganoSeg.



The most recent image adjustment is undone by pressing ‘z’, but if the last thing clicked was a GUI component (e.g., checkbox, button, listbox), you must click somewhere on the GUI that is not a clickable component to reorient the app to detect keyboard actions.

Chapter 4: Image Grouping

For certain analyses, it may be useful to group images based on the contents of their filenames. OrganoSeg2 groups images to facilitate grouped exports (chapter [11](#)) or batch tracking (chapter [13](#)) of images. By selecting the tab at the bottom of the GUI that says “Grouping Options”, users may enter a regular expression (regex) that will classify each image based on key variables in the filename. Key variables are declared using the format (?<VARIABLE_NAME>REGEX), where REGEX is a sequence of characters that uses regex notation to match the part of the filename of interest.

For example, using the regex...

```
^(?<Date>.*)_scan(?<Case>.*)_rad_(?<StartDate>.*)_Plate_(?:D|R)_p00_0_(?<WellNumber>[A-Z][0-9]{2})f00d(?<ChannelNum>[0-9]).*
```

and the filename ...

20241220_scan149_rad_20241218_Plate_R_p00_0_C04f00d4.TIF

the following key variables are captured...

Date = “20241220”, Case = “149”, StartDate = “20241218”, WellNumber = “C04”, ChannelNum = “4”

The notation “.” used for many of the variable names means any character (.) appearing 0 or more times (*). You may use the constant portions of the filename (e.g., “scan”, “p_00_0”) as place holders to separate the variables of interest. However, if desired, you may enter a more specific regex. For example, “[A-Z][0-9]{2}” means 1 character between A and Z (case-sensitive), followed by 2 numeric characters. The expression (“D|R”) means that you want to match either the letter “D” or “R”, but because there is “?:”, it means that you do not want to capture the token. For more information on regex formatting, see the MATLAB documentation for [regexp](#), or other online resources.

Once an expression is entered, a table will populate where each row is a variable name, and each column is an option for grouping, which may be used for grouped exports or batch tracking. See chapters [11](#) and [13](#) for more information. You can verify the successful classification of each image by selecting the “Groups” tab above the image listbox, which will display a table of classifications for each image.

Manage Images

Image Selection

Images	Groups
Date	Case
20241220	149
20241220	149
20241220	149
20241220	149

Segment

Num Workers: 0

Segment Current (s) | Apply to All

Select Images to Segment

Export

Current | Export Set

Export in groups

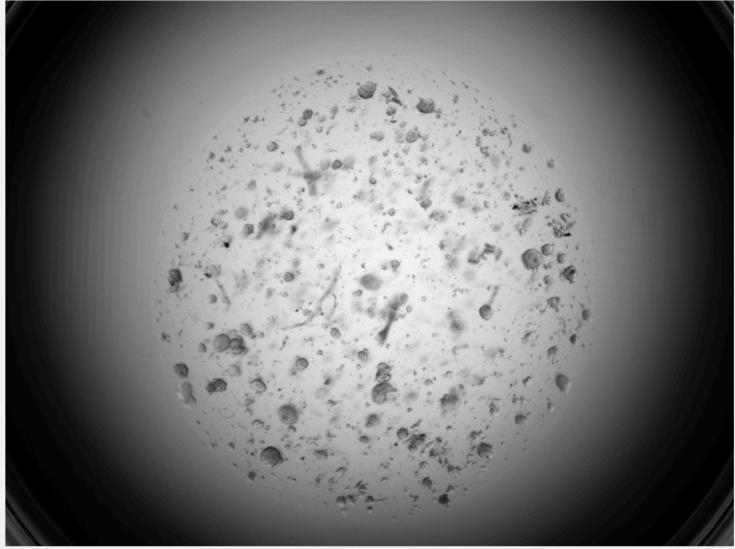
Select Images to Export

Selected Metrics: All metrics

No Classifications: Include

Raw Grayscale Image

Crop (x) Brightness Rotate Revert

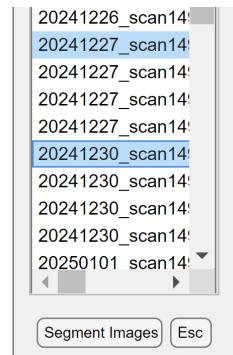


Change Classification ▾ Remove (r) Combine (c)

Segment Options		Grouping Options	
Regex: ^(?<Date>.*)_scan(?<Case>.*)_rad_(?<StartDate>.*)_Plate_(?:D R)_p00_0_(?<Well>.*)			
Group	UseInGrouping	Key	MatchOrContains
Date	<input type="checkbox"/>		Exact Match ▾
Case	<input type="checkbox"/>		Exact Match ▾
StartDate	<input type="checkbox"/>		Exact Match ▾
WellNumber	<input type="checkbox"/>		Exact Match ▾
ChannelNum	<input type="checkbox"/>		Exact Match ▾

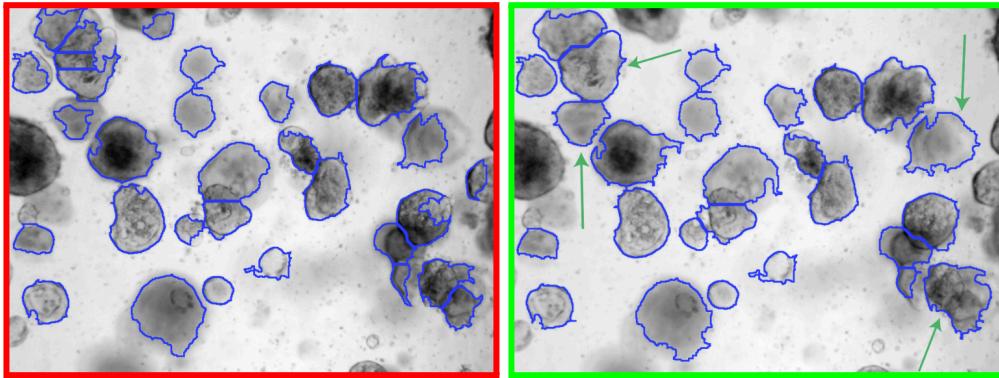
Chapter 5: Segmentation

To segment images, first set the desired parameters, which are described in detail below. To segment only the current image, select “Segment Current” (or press “s”). To segment all images with the parameters currently selected, press “Apply to All”. To segment only a subset of the images, first select “Select Images to Segment”, which will expand the image list and enable multiselection. Use Ctrl and Shift to select the desired images, and then press “Segment Images”.



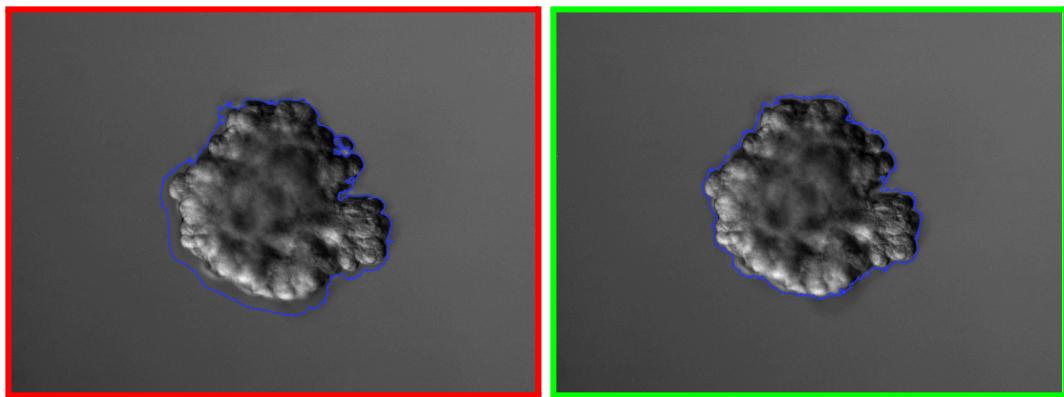
Segmentation Corrections

Out-of-Focus Correction (default: ON):



This option performs multi-window thresholding to include blurred content outside of the image plane. The red outlined image displays results with the option OFF; the green outlined image displays results with the option ON. Arrows highlight spheroids that improve upon out-of-focus correction.

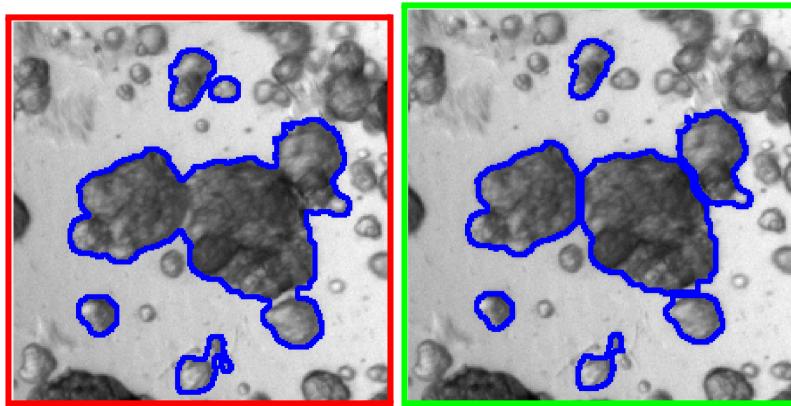
DIC Correction (default: OFF):



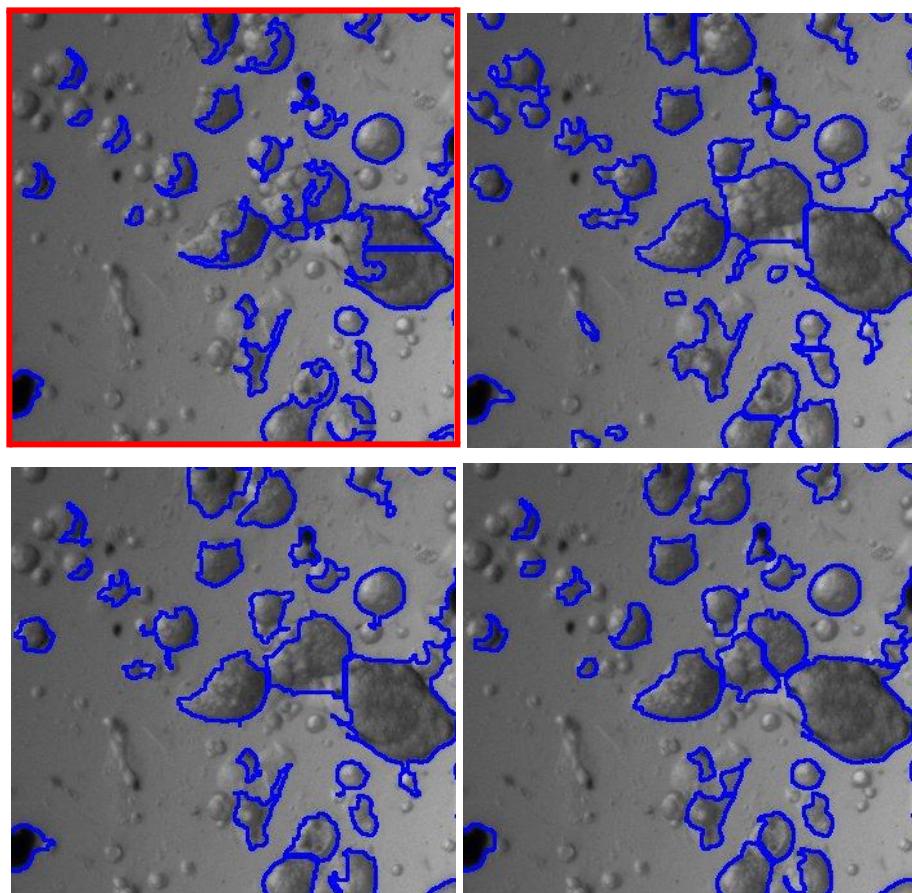
This correction allows for a tighter identification of the border in DIC images. The red outlined image shows DIC Correction OFF, and the green outlined image shows DIC correction ON.

Watershed Split (default: OFF):

This option splits segmented objects along “watersheds” to separate distinct objects.



Edge Correction (default: OFF):



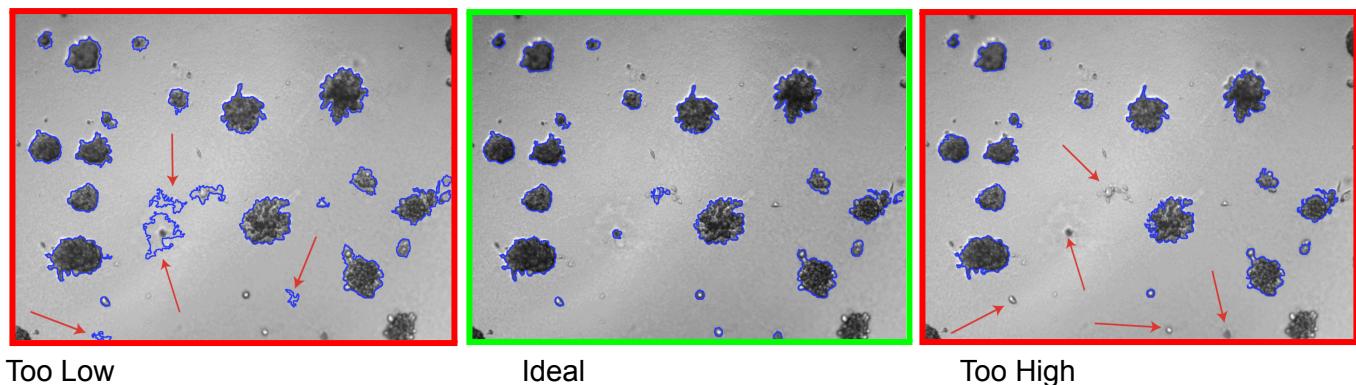
This option uses the gradient function on pixel intensity in the image to identify

boundaries of objects. It uses the edge intensity parameter (described below) to determine the minimum gradient value that is added to the segmentation. Users choose between edge correction using “Gradient Only” (top right left), “Gradient Preserve Boundary” (bottom left), and “Gradient + Watershed” (default - bottom right). The first option uses the original segmentation to maintain separation between objects that were segmented separately without correction, and it does not allow for new segmented objects. The second option is the least limited and allows for the combination of objects or the segmentation of new objects. The final option also preserves separation between objects and uses a watershed transformation to identify the most apparent boundary in the image gradient region of the spheroid. This choice is made under Preferences → Edge Correction.

Segmentation parameters

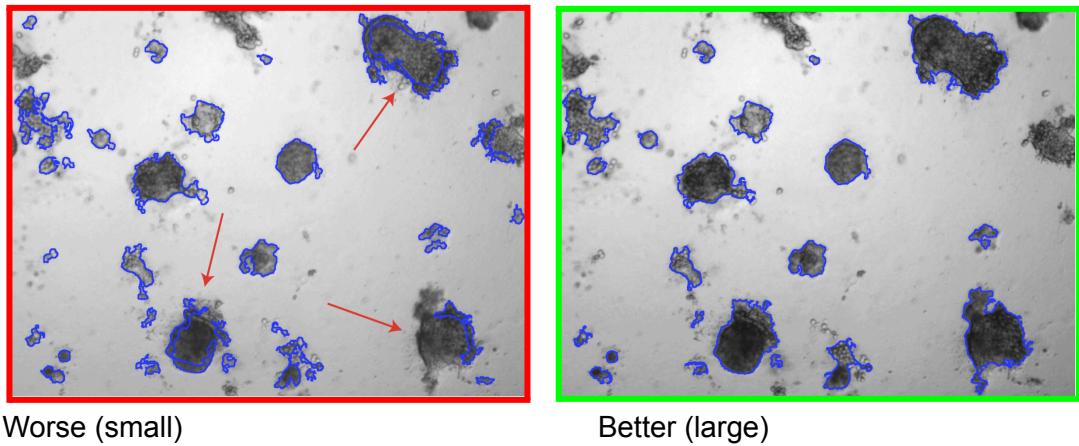
Intensity threshold (default: 1 [no modification]):

This slider adjusts a parameter that modulates the Otsu threshold level. Lower thresholds distinguish finer contrast differences, while a higher threshold requires higher contrast differences to identify an object. The ideal intensity threshold varies from image to image.



Window size (default: 100):

The window size parameter determines the averaging filter in the adaptive thresholding algorithm. Larger window sizes capture more global detail; smaller window sizes capture more local detail. Ideal window size varies from image to image.



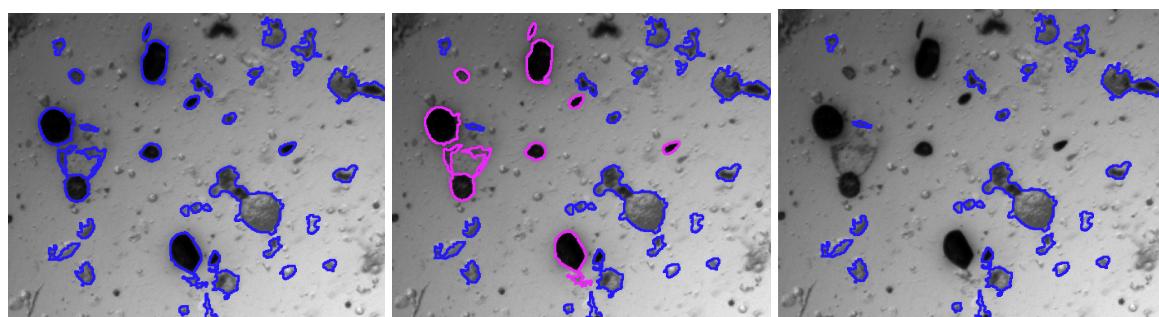
Size threshold (default: 100):

This parameter eliminates any objects under the specified pixel area value.

Edge Intensity (default: 0.3 - only impacts segmentation if Edge Correction is selected, and if either “Gradient Preserve Boundary” or “Gradient Only” are selected in Preferences → Edge Correction. Since the default edge correction is “Gradient + Watershed”, this option is disabled by default, and the edge correction style must be changed to enable it): This parameter adds pixels to the segmentation based on the gradient of pixel intensity. The edge intensity value is multiplied by the approximate background intensity for each pixel, which is then used to threshold the gradient magnitude. A smaller value requires less distinct changes in pixel intensity and will segment more. If the value is too low, the segmentation will spread to the image boundary and will not show up because image boundaries are cleared during segmentation.

Contaminant Intensity (default: 0)

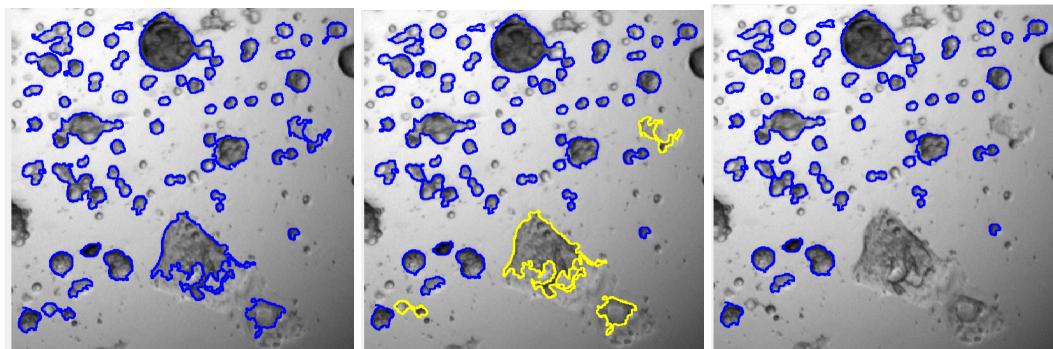
This parameter marks segmented objects as possible contaminants, where any object whose mean pixel intensity is lower than this value will be highlighted in magenta and stored for further modification. Higher values will mark more objects. If zero, the parameter has no effect. Marked objects will still be included in the segmented data.



Contaminant Intensity = 0 Contaminant Intensity = 0.25 Magenta segments removed

Minimum Circularity (default: 0)

This parameter marks segmented objects as non-circular; where any object whose circularity is lower than this value will be highlighted in yellow and stored for further modification. Higher values will mark more objects. If zero, the parameter has no effect. Marked objects will still be included in segmented data.

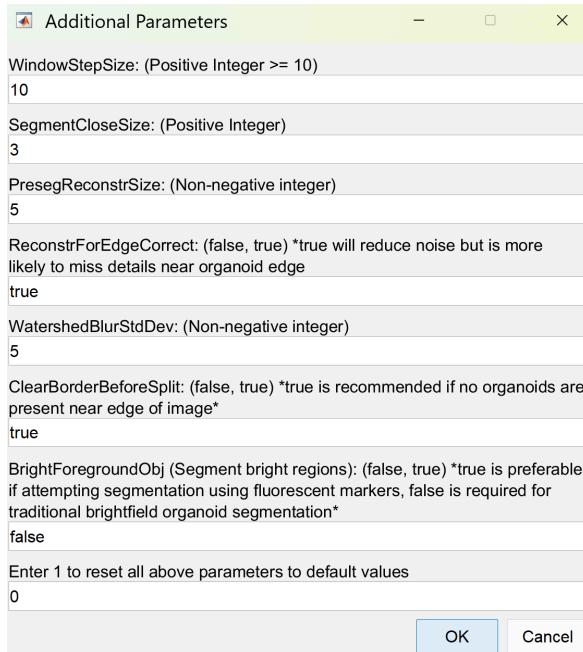


Minimum Circularity = 0 Minimum Circularity = 0.3 Yellow segments removed

Using the above features, modify the segmentation until the results are satisfactory for the application. Press “Segment Current (s)”, or press ‘s’ on the keyboard to segment the current image. If multiple images contain organoids with similar morphologies, press "Apply Settings to All" to use the current settings for the remainder of the images, or press “Segment Selected Images” and choose the images from the drop-down that you would like to apply the settings to. After applying settings, additional fine-tuning of individual images is possible by clicking on an image in the “Selected Image” list and altering its segmentation parameters.

Additional Parameters

The additional parameters tab (Preferences → Additional Parameters) allows the user to change selected values used in segmentation, which are not included in the main parameters on the GUI.

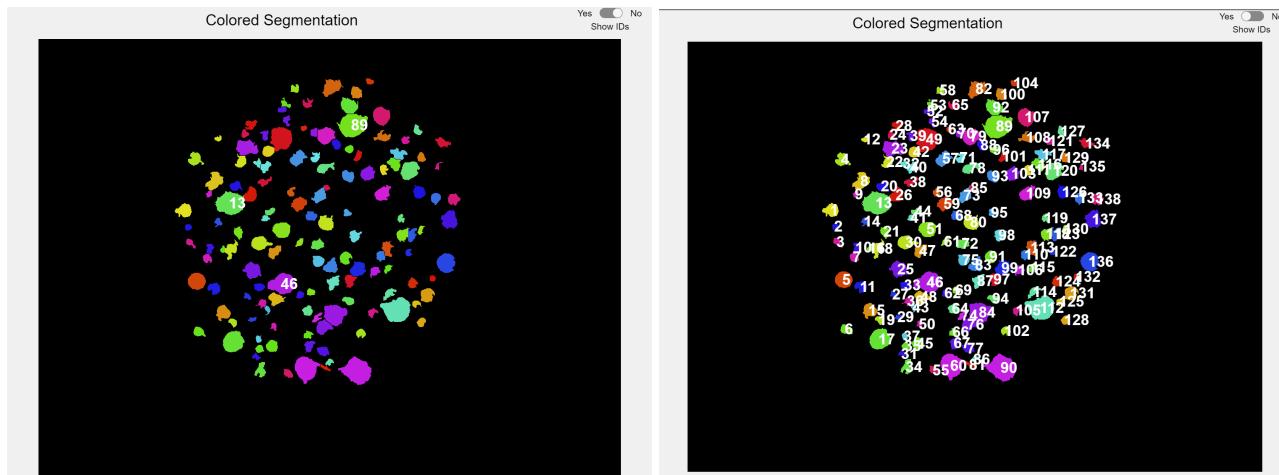


Internal Parameter	Description	Default Value	Practical Range of Values
WindowStepSize	Iterative decrease (in pixels) for the window size used for adaptive thresholding when performing out-of-focus correction. Larger values increase segmentation speed with possibly some loss of accuracy.	10 pixels	10 pixels–window size
SegmentCloseSize	Size of the image close (dilation and then erosion, in pixels) applied to segmentation masks. Larger values provide more edge smoothing and thus less edge detail.	3 pixels	1–10 pixels
PresegReconstrSize	Size of the structuring element (in pixels) used to create a marker image for morphological reconstruction of the original image before segmentation. Larger sizes remove larger background fluctuations in image intensity but reduce the detail of organoid edges.	5 pixels	1–10 pixels
UseReconstrForEdge	Logical indicating whether to use the reconstructed (true) or original (false) image for gradient thresholding during edge correction. True reduces artifacts and noise near organoid boundaries but decreases sensitivity for restoring organoid edges.	True	True or false
WatershedBlurStdDev	Standard deviation of the Gaussian filter applied to the distance-transformed segmentation mask during watershed splitting. Smaller values increase splitting sensitivity but also splitting artifacts.	5 pixels	1–10 pixels
ClearBorderBeforeSplit	Logical indicating whether the border is cleared before and after splitting (true) or only after splitting (false). True excludes artifacts that do not appear in the unsplit segmentation, as well as some organoids that are fully contained in the image but near the border.	True	True or false
BrightForegroundObj	Logical indicating whether foreground objects are darker (false) or lighter (true) than the background. False segments relate to brightfield images, whereas true segments relate to fluorescence images.	False	True or false

Chapter 6: GUI layout

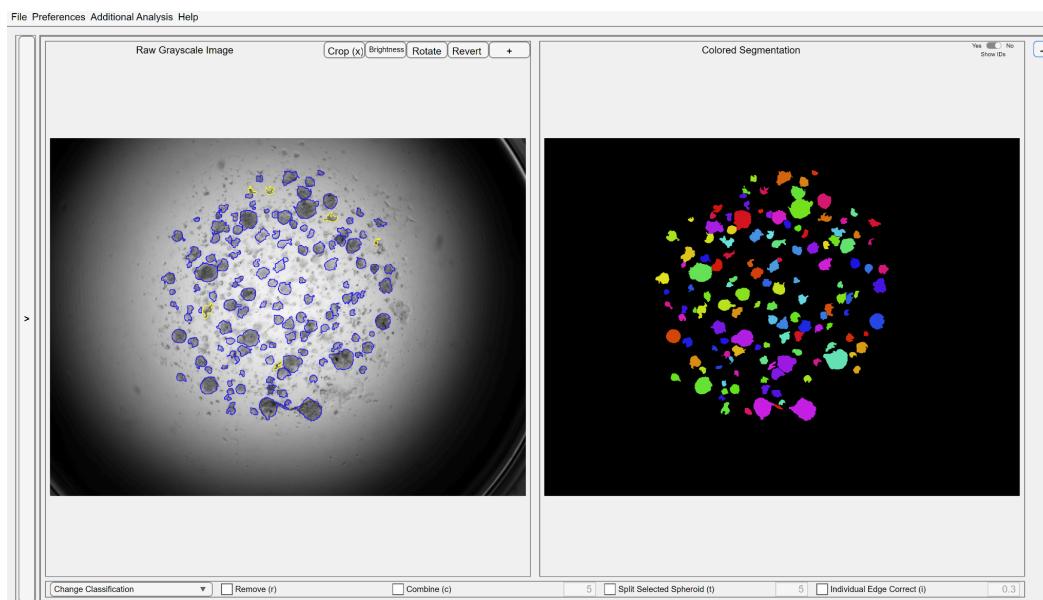
After segmenting, a perimeter overlay will appear in the “Raw Image” panel, and a color-labeled image will appear to its right in the “Colored Segmentation” panel. Below the color-labeled image, a zoom-in of the highlighted spheroid will appear. Scrolling through the “Selected Spheroid” list will show a close-up of each spheroid in the image.

The color-labeled image originally appears without numbers identifying the segmented objects. To show the segmented ID, either click on an individual organoid, or set the “Show IDs” switch to “Yes”

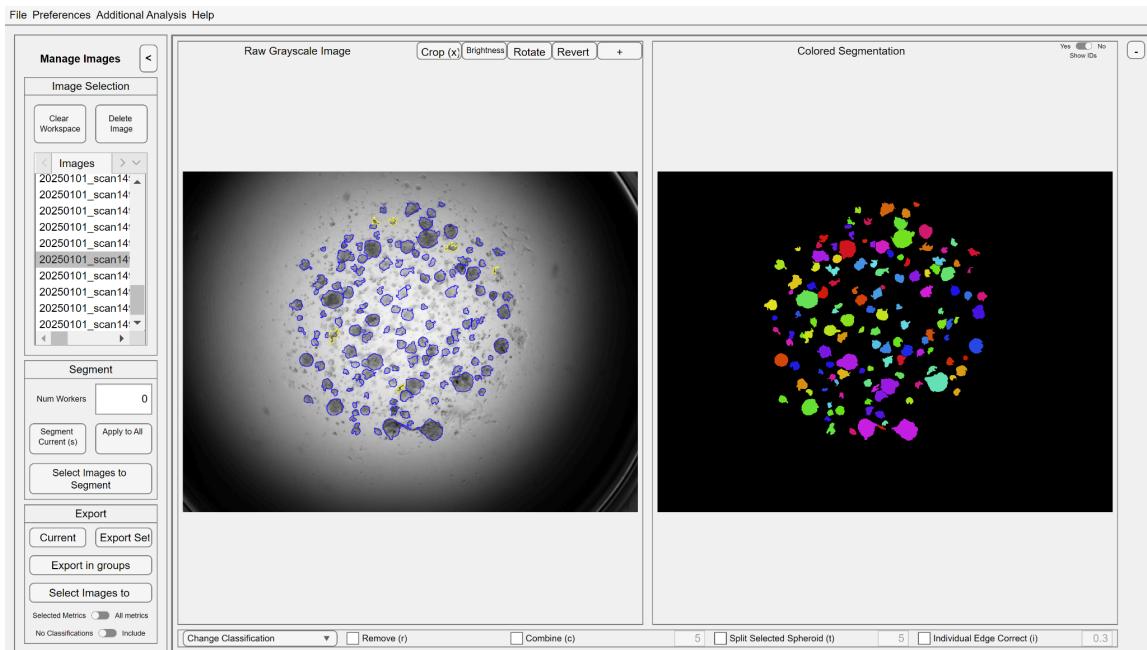


Panel Resizing:

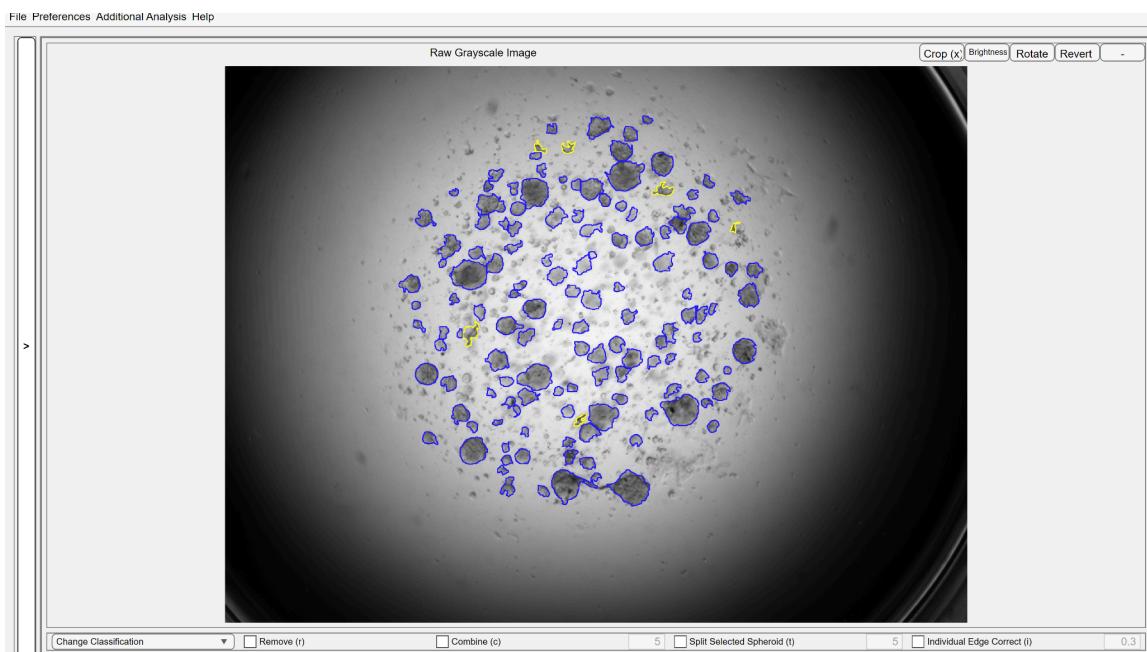
- Selecting the ‘+’ button in the top right corner will maximize the view of the segmented images.



- The ‘>’ in the left panel expands the “Manage Images” panels so that the user can go between images, as well as segment and export images. Alternatively, the up and down arrows on the keyboard move between images, and pressing ‘s’ segments the current image. If the panel is open, selecting ‘<’ will collapse it.



- The ‘+’ inside the “Raw Grayscale Image” panel maximizes the raw grayscale image to fill the entire space, while still presenting the spheroid editing toolbar so that users can manually adjust the segmentation.

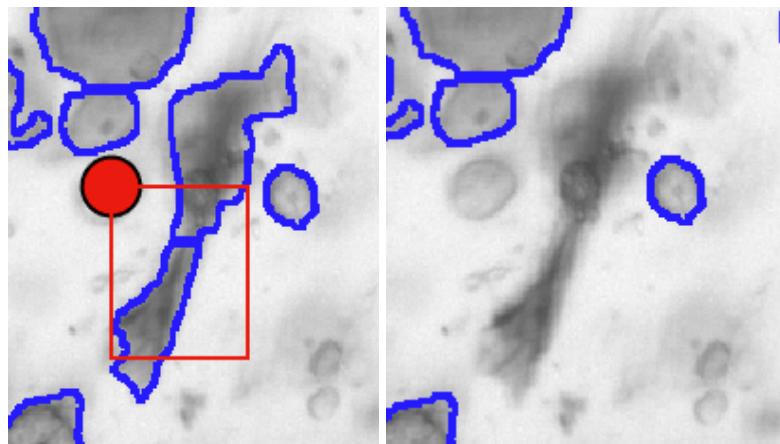


Chapter 7: Spheroid Editing Toolbar

For all actions on the spheroid editing toolbar, keyboard shortcuts are available, but if the last thing clicked was a GUI component (e.g., checkbox, button, listbox), you must click somewhere on the GUI that is not a clickable component in order to reorient the app to detect keyboard actions. You may escape any tool without performing the action by pressing ‘e’ (be sure to click somewhere on the GUI if pressing ‘e’ does not work).

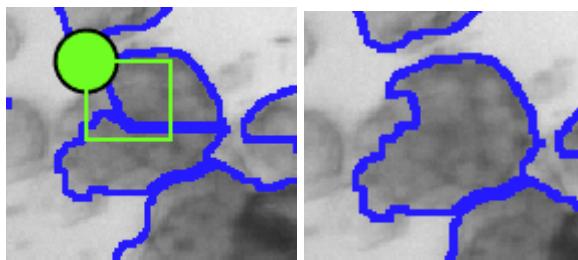
Most actions on the spheroid editing toolbar will disable all actions in the app outside of those intended by the action. To escape using the tool, either reselect the checkbox or press “e” (reorienting as described above if necessary).

Remove: Remove spheroids in a selected region. Select the Remove checkbox or press “r” on the keyboard. Then, select two corners of a rectangle and press Enter to remove any segmented objects inside that region.



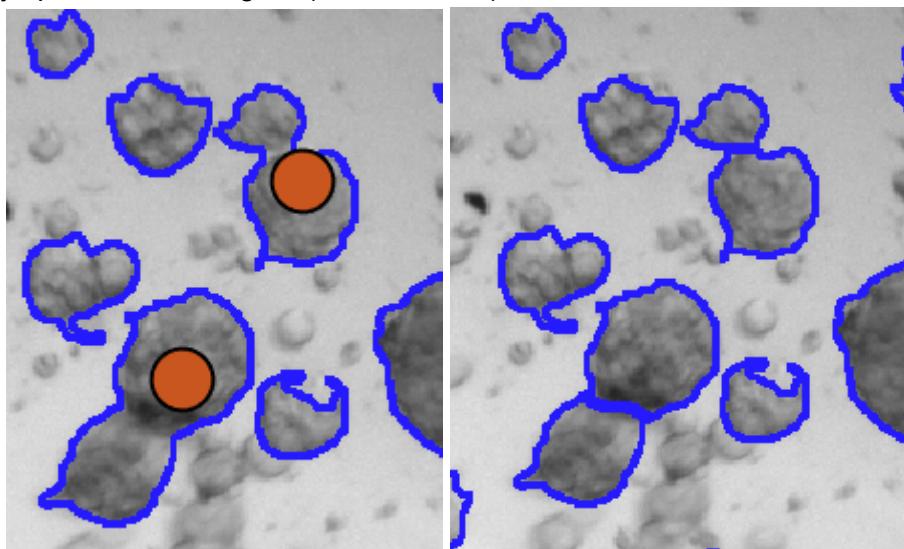
Combine: Join two spheroids into one connected component. Select the Combine checkbox or press “c”. If desired, change the value in the edit field to the right to change the closing degree (described in Chapter 5, Additional Parameters), where larger values combine objects with greater separation. Then, select two corners of a rectangle to combine all segmented objects inside that region. If all objects inside the rectangle cannot be joined into one connected object, the combine action will not take place.

The default gap should join spheroids that are falsely split, but if objects are further apart, a larger close may be necessary. If needed, change the value to the right of the checkbox to change the size of the image close that connects the objects.



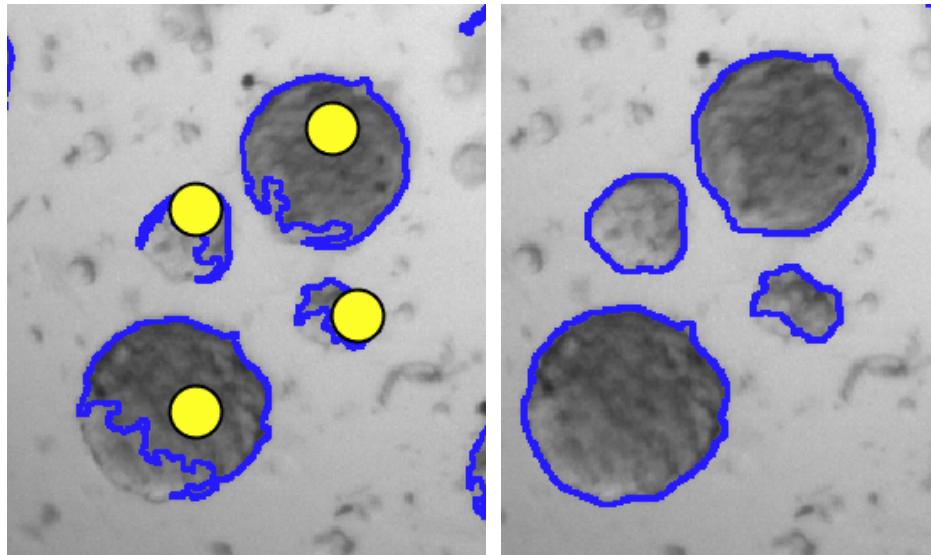
Split Selected Spheroid: Apply watershed transformation on individual spheroids. Select the Split Selected Spheroid checkbox, or press “t”. If desired, change the value in the edit field on the right to change the watershed blurring standard deviation (described in Chapter [5](#), Additional Parameters). Click on all segmented objects that you wish to split, and press Enter.

Individual splitting may be applied to previously unsplit images or to images that were already split but with a higher (less sensitive) watershed standard deviation.



Individual Edge Correct: Apply edge correction using the image gradient to selected spheroids. Select the Individual Edge Correct checkbox, or press “i”. If desired (and not using “Gradient + Watershed” correction), change the value in the edit field on the right to change the sensitivity of edge correction (described in Chapter [5](#), Segmentation Parameters).

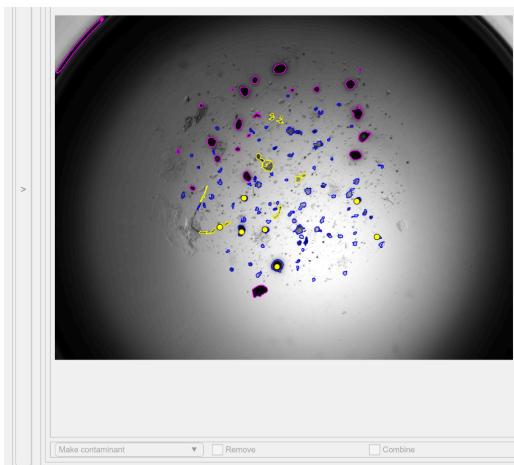
Individual edge correction may be applied to previously uncorrected images, or to images that were already corrected but with a higher (less sensitive) edge correction threshold.



Change Classification: Mark spheroid as a contaminant and/or non-circular, or as a regular spheroid if previously marked into one of those categories. The action also allows for bulk removal of spheroids with these classifications, which may be done regardless of whether or not the “Contaminant Intensity” or “Minimum Circularity” parameters are set.

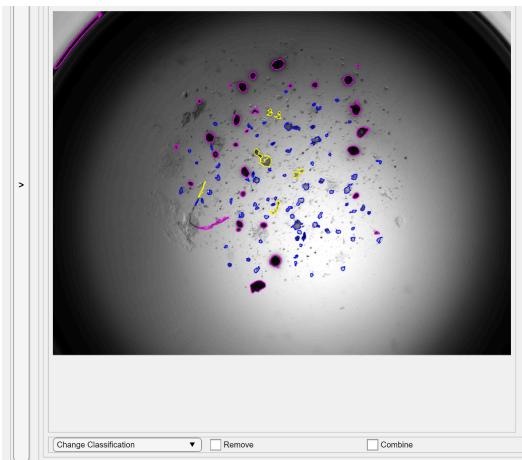
To change classification (If only removing, skip to 4):

1. Click on the “Change Classification” drop-down and choose either “Make spheroid” (or press “1”), “Make contaminant” (or press “2”), or “Make non-circular” (or press “3”). “Make spheroid” removes the contaminant and/or non-circular designation from a given spheroid.
2. Click on all spheroids to reclassify with the given classification. They will be marked with dots.

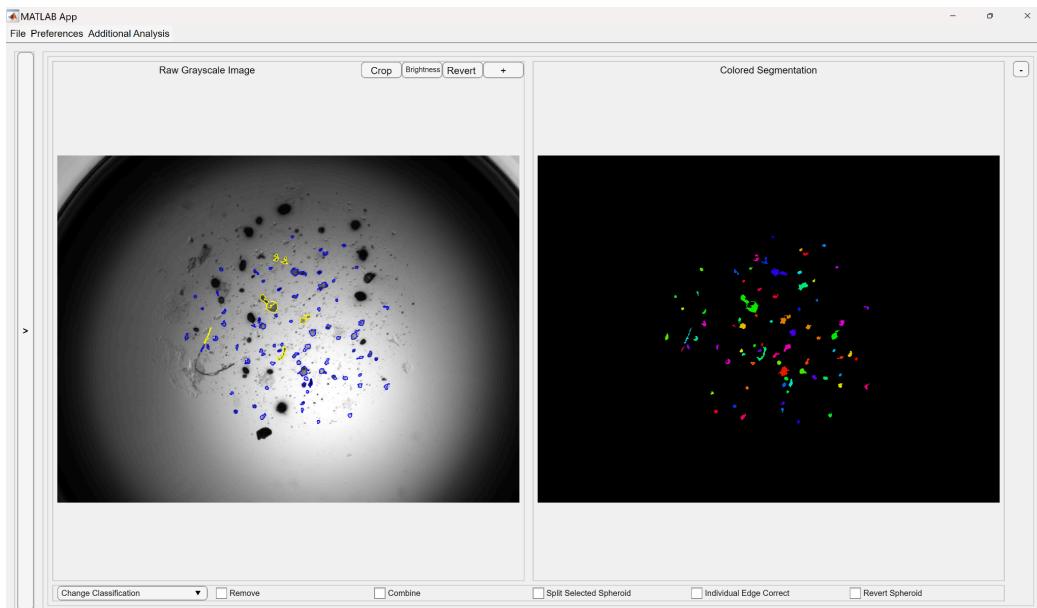


3. Press Enter to change the classification. The selected spheroids should be marked magenta if changed to a contaminant, yellow if changed to non-circular, and

blue if changed to a spheroid. (If an object is labeled as a contaminant and non-circular, it will only appear magenta).



4. To remove all contaminants or non-circular objects, select either “Remove Contaminants” (or press “4”) or “Remove non-circular objects” (or press “5”), and press Enter.



Change classification may also be used to remove several objects at once that are not confined to one region. Reclassify all desired objects as contaminants, and then perform bulk removal on the objects.

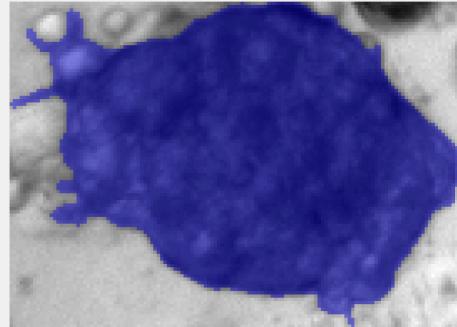
Undo (File → Undo or “z”): This undoes the most recent change to the current image’s data and restores segmentation data from before any of these tools were performed. The previous data are saved for all images, so undo actions are possible on an image after leaving and coming back.

Chapter 8: Metric Selection Tool (Preferences → Metrics)

The user may select as many of the metrics to export as needed. The default metrics are: area, perimeter, circularity, diameter, and eccentricity. Click on the checkbox of any metric with a blue colored description to view a visual representation in the right panel. The selected metric description will be highlighted in red.

Metric	Description
<input checked="" type="checkbox"/> Area	Number of pixels in segmented region
<input checked="" type="checkbox"/> Perimeter	Distance around boundary of segmented region
<input type="checkbox"/> Perimeter to Area Ratio	Ratio of perimeter divided by area
<input type="checkbox"/> Circularity	Roundness of segmented region
<input type="checkbox"/> Diameter	Diameter of a circle with same area as segmented region
<input checked="" type="checkbox"/> Eccentricity	Ratio of the distance between foci of ellipse and major axis length
<input type="checkbox"/> Maj. Axis Length	Major axis length of ellipse with same second central moments as segmented region
<input type="checkbox"/> Min. Axis Length	Minor axis length of ellipse with same second central moments as segmented region
<input type="checkbox"/> Convex Area	Number of pixels in convex hull (smallest convex polygon containing segmented region)
<input type="checkbox"/> Extent	Ratio of pixels in segmented region to pixels in total bounding box
<input type="checkbox"/> Orientation	Angle between x-axis and major axis
<input type="checkbox"/> Solidity	Ratio of area to convex area
<input type="checkbox"/> Z	Imaginary component of complex Zernike moment of 4th order shape
<input type="checkbox"/> A	Amplitude of complex Zernike moment of 4th order shape
<input type="checkbox"/> Phi	Angle of complex Zernike moment of 4th order shape
<input type="checkbox"/> Mean Pixel Intensity	Average pixel intensity in segmented region
<input type="checkbox"/> Std of Pixel Intensity	Standard deviation pixel intensity in segmented region
<input type="checkbox"/> C.V. of Pixel Intensity	Coefficient of Variation of pixel intensity in segmented region
<input type="checkbox"/> Skewness	Skewness of pixel intensity in segmented region
<input type="checkbox"/> Kurtosis	Kurtosis pixel intensity in segmented region
<input checked="" type="checkbox"/> Contrast	Intensity contrast between pixel and neighbor (i.e., variance)
<input checked="" type="checkbox"/> Correlation	Correlation of pixel and neighbor
<input checked="" type="checkbox"/> Energy	Dominance of adjacent pixel combinations
<input checked="" type="checkbox"/> Homogeneity	Likelihood of adjacent pixels being equal (i.e., smoothness)

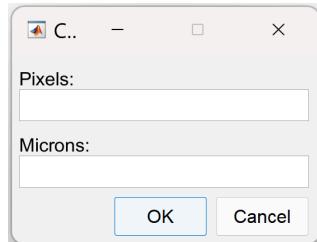
Visual Representation



Area

Chapter 9: Pixel Calibration Tool (Preferences → Calibration)

The user may choose a conversion from pixels to micrometers (μm). The micron-to-pixel ratio will be applied to all exported images in the set.



Chapter 10: Save Images (File → Save)

Current Images

Raw-overlay and Colored Images:

Exports the perimeter overlay and color-labeled segmentations as shown in the top row of OrganoSeg2

Cropped Spheroid:

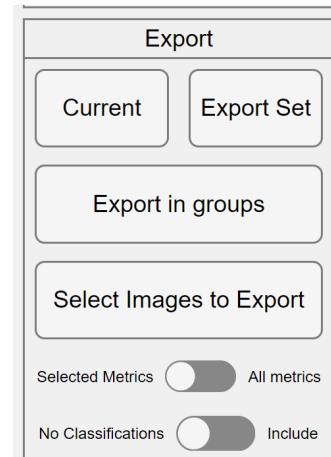
Exports the zoomed image of the selected spheroid as shown in the bottom right corner of OrganoSeg2

Image Set:

Exports the raw-overlay and color-labeled segmentations for all images into the selected folder.

Chapter 11: Export

Under the “Export” panel, select either “Current” or “Export Set”, or “Select Images to Export” to export the respective metric signature. A save window will appear. Choosing *.mat will save metrics, OrganoSeg parameters (Otsu threshold, Max-window size, size-exclusion threshold), images, and processed intermediates, allowing for later modification or referencing of the same image set (see Chapter [10](#)). Choosing *.xls or *xlsx will save only metrics. After exporting, the workspace remains populated for further modification if desired.



Seven metrics are exported with the default settings: Area, Perimeter, Eccentricity, Contrast, Correlation, Energy, and Homogeneity. Metrics may be added or removed with the “Metric Selection Tool” (Preferences → Metrics) as described in Chapter [7](#).

Selected Metrics, All Metrics Toggle (default: selected metrics): Choosing All Metrics will include all metrics in export data without having to select each one in the Metrics Selection Tool, and choosing Selected Metrics will refer to those selected as described above.

Export in groups: Selecting this option will apply the classifications made using regex parsing of image filenames (Chapter [4](#)). To use this option, you must first select which variables you want to group by, using the “Grouping Options Tab” in the bottom panel. “Group” refers to each of the variables in your filename. Selecting “UseInGrouping” will split all images based on whether they have equal values of that variable. You may select multiple variables to separate combinations of variables. If you only want to export a subset of the images, use the “Key” field to define that subset. The “MatchOrContains” field determines whether that key must be an exact match or just contained in the variable name. The Key field will only be used if that variable is selected to “UseInGrouping”.

For example, if you have images that match the following groups:

“Case=149, WellNumber=C03”
“Case=149, WellNumber=D03”

“Case 149, WellNumber=C04”
“Case 150, WellNumber=C04”

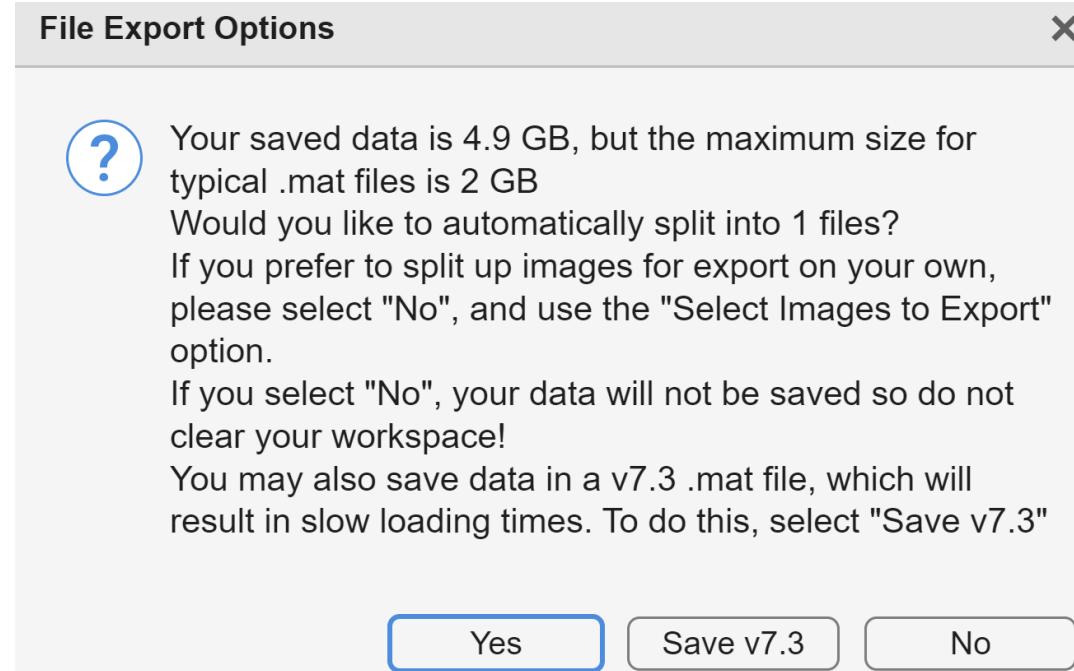
The selection on the left would produce four separate files, one for each group. Meanwhile, the selection on the right only produces two files, one for “Case=149, WellNumber=C03” and one for “Case 149, WellNumber=C04.” The export file name will use your selected file name as the prefix, and append the group conditions (e.g., groupedExport_149_C04.xls)

Segment Options		Grouping Options	
Regex ^(?<Date>.*)_scan(?<Case>.*)_rad_(?<StartDate>.*)_Plate_(?:DIR)_p00_0_(?<Well>.*)		Regex ^(?<Date>.*)_scan(?<Case>.*)_rad_(?<StartDate>.*)_Plate_(?:DIR)_p00_0_(?<Well>.*)	
Group	UseInGrouping	Key	MatchOrContains
Date	<input type="checkbox"/>	Exact Match	Exact Match
Case	<input checked="" type="checkbox"/>	Exact Match	Exact Match
StartDate	<input type="checkbox"/>	Exact Match	Exact Match
WellNumber	<input checked="" type="checkbox"/>	Exact Match	Contains
ChannelNum	<input type="checkbox"/>	Exact Match	Exact Match

Segment Options		Grouping Options	
Regex ^(?<Date>.*)_scan(?<Case>.*)_rad_(?<StartDate>.*)_Plate_(?:DIR)_p00_0_(?<Well>.*)		Regex ^(?<Date>.*)_scan(?<Case>.*)_rad_(?<StartDate>.*)_Plate_(?:DIR)_p00_0_(?<Well>.*)	
Group	UseInGrouping	Key	MatchOrContains
Date	<input type="checkbox"/>	Exact Match	Exact Match
Case	<input checked="" type="checkbox"/>	149	Exact Match
StartDate	<input type="checkbox"/>	Exact Match	Exact Match
WellNumber	<input checked="" type="checkbox"/>	C	Contains
ChannelNum	<input type="checkbox"/>	Exact Match	Exact Match

Large .mat Exports (> 2GB): You may not save a .mat file with greater than 2 GB. If you do so, you will be prompted with a message asking if you would like to automatically split the .mat file into smaller sections. If you select "Yes", your .mat files will be named using your chosen filename as a prefix, followed by indices for each export (e.g., largeExport_1.mat, largeExport_2.mat, largeExport_3.mat). If you select "No," you will be returned to the main screen without any data saved. You may also choose to save the .mat file using version 7.3, which MATLAB warns will have slower running times, but will still correctly save and reload your data.

- It is possible that the split file will still be over 2 GB if the amount of storage is unevenly distributed across images. The software tries to avoid this by splitting conservatively, but make sure to check for this message to appear again before all exports complete.



Chapter 12: Loading Segmented Images

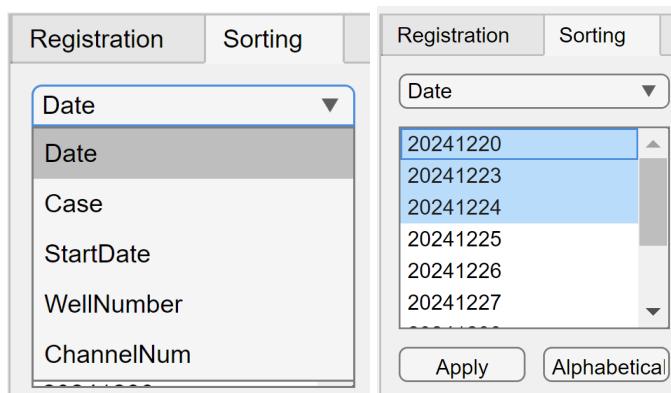
Previously Segmented Images (File → Open → Previously Segmented Images)

The user may select one or multiple .mat files of previously segmented images by OrganoSeg (see Chapter [10](#)). To select multiple images, use Ctrl and Shift as used for normal file selection. The raw image, colored segmentation image, and spheroid zoom will be displayed for each image in the stack as they were saved, and segmentations can be further modified if desired. Edits will not affect the save .mat file, unless exported to the same .mat file, in which case the data will be overwritten.

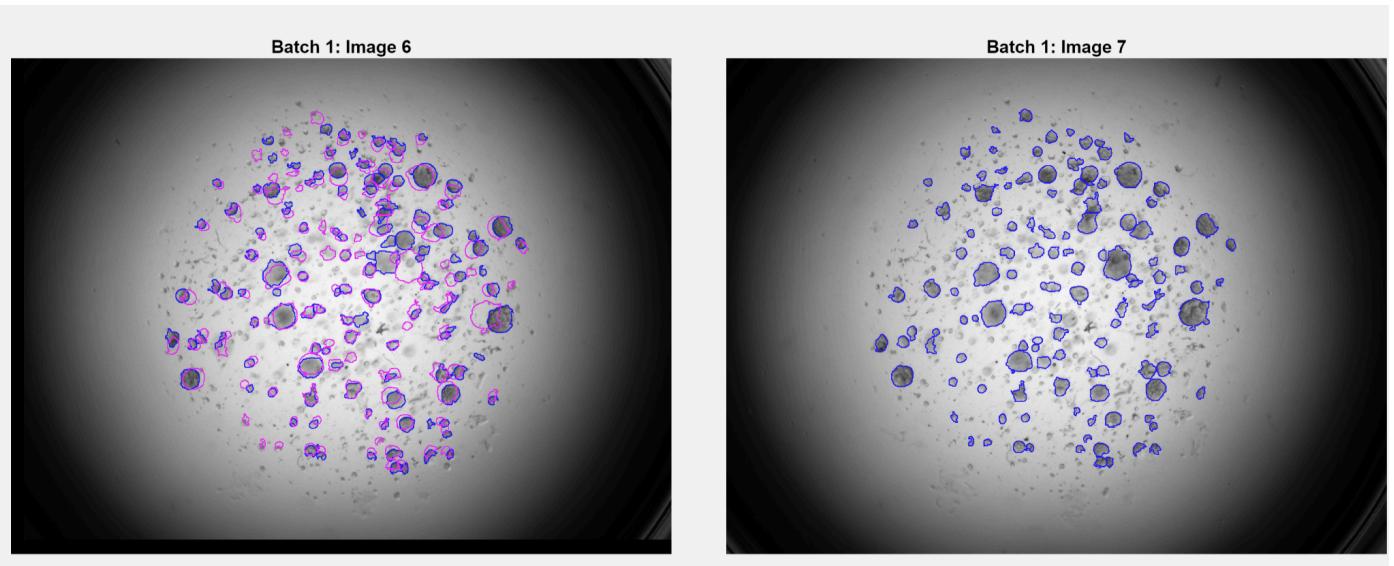
Chapter 13: Tracking Analysis

After segmenting images of the same wells longitudinally, users have the option to perform tracking analysis to connect data across images to the same organoid.

1. Segment brightfield images and modify as desired
2. Go to the tracking menu (Additional Analysis → Tracking).
 - a. If you have grouped your images using a regex expression, you may track multiple longitudinal image sets in the same window, with those images separated in batches based on the grouping (see Chapter 4, Image Grouping and Chapter 11, Export in groups for more information about how grouping works). From the tracking menu, select “Track in Batches.” Use multi-select controls to select the images you would like to include in the analysis. If you select an image that does not fall under your grouping criteria, it will not be included in the tracking analysis. Press “Begin Tracking”
 - b. If you have not grouped images, you may only track one longitudinal set at a time. Select “Track Single Batch,” and then select **IN ORDER** the images you wish to analyze. You will not be able to change the order after this point.
3. If necessary, sort images by going to the sort tab in the top left. The drop-down will populate with all the variables from your filenames. Select the variable you would like to sort by. The list will populate with the list of those variable values for the first batch of images. If you wish to sort, you should only include image sets that have the same number of images and values for the sorting variable (i.e., the same time points). If the values should be arranged in alphabetical order, select the “Alphabetical” button. Otherwise, multiselect all items in the list in the order desired, and then select “Apply.” All images within each batch should then be sorted in the desired order. This option is not available if “Track Single Batch” was used.

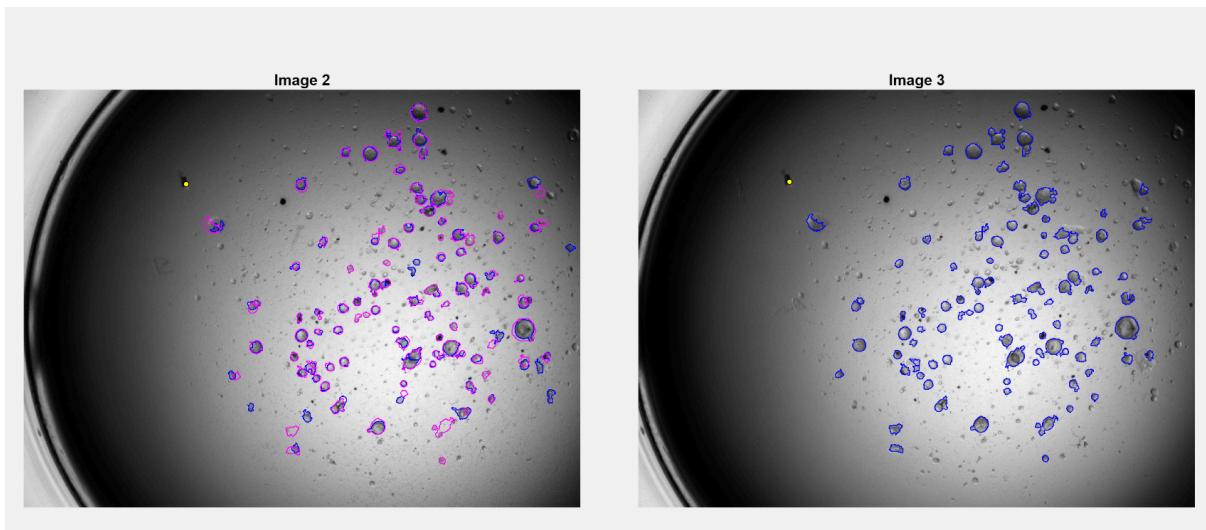


4. On the “Registration” tab, use the drop-down to select the method of image registration you wish to use. The default is rigid registration without rotation (only translation), and the other options are rigid registration with rotation and non-rigid registration. Select “Register Batch” to test the registration on a single batch, or “Register All” to register all batches. Once registration is complete, the registered perimeter of the right image segmentation will be displayed on top of the left image. You may select different images in each batch to see how they register relative to each other.

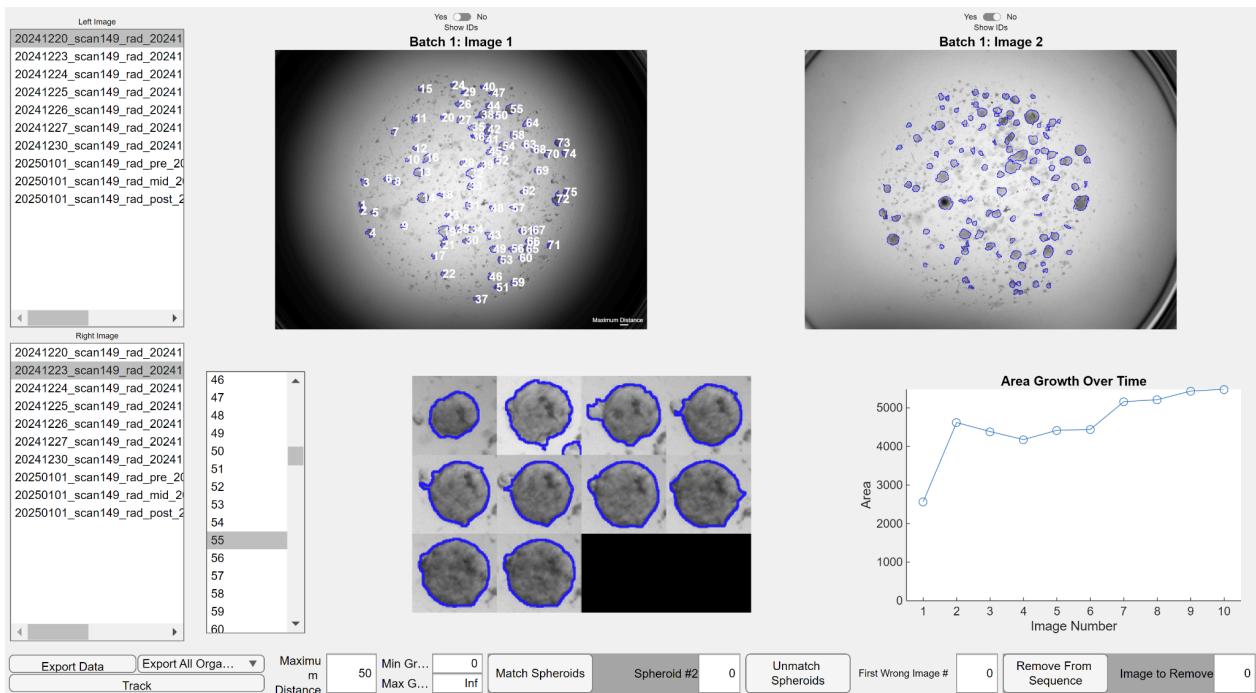


- a. The two numerical parameters only apply to non-rigid registration. The “Number of Iterations” will improve performance at the expense of higher run time. For “Degree of Smoothing”, lower values allow for more deformation, while higher values become closer to a rigid registration.
- b. The switch between “Use Segmentation Mask” and “Use Raw Images” determines the basis for registration. Segmentation mask is generally recommended, but the raw image may be useful if there is low organoid density or contaminants that are inconsistently segmented.
- c. The Num Workers option allows for parallelization. This is recommended if you are registering several batches at once (especially using non-rigid registration), but otherwise may be slower than running without parallelization. Enter a maximum of 4 for this value if parallelization is desired.
- d. Rigid registration with rotation and non-rigid registration are only recommended if the nature of the images requires it. Non-rigid registration may be helpful if there is noticeable movement of organoids in the culture. However, these options are slower and they are more likely to misalign the whole image.
- e. For “Track Single Batches”, a fourth option of manual translation is available if none of these automated options work. For manual translation, select one point on each of the two images shown that correspond to the same point in your culture. An overlay of the right image segmentation will be displayed on the left. Once satisfied with the alignment, select “Confirm Orientation” or press the ‘Enter’ key, which will move to the next image, retaining the point that was previously selected. To choose new reference points, either click a different point or “Reselect Base Point(s)”, which will clear the overlay. The reference points do not have to be the same for each pair of images.

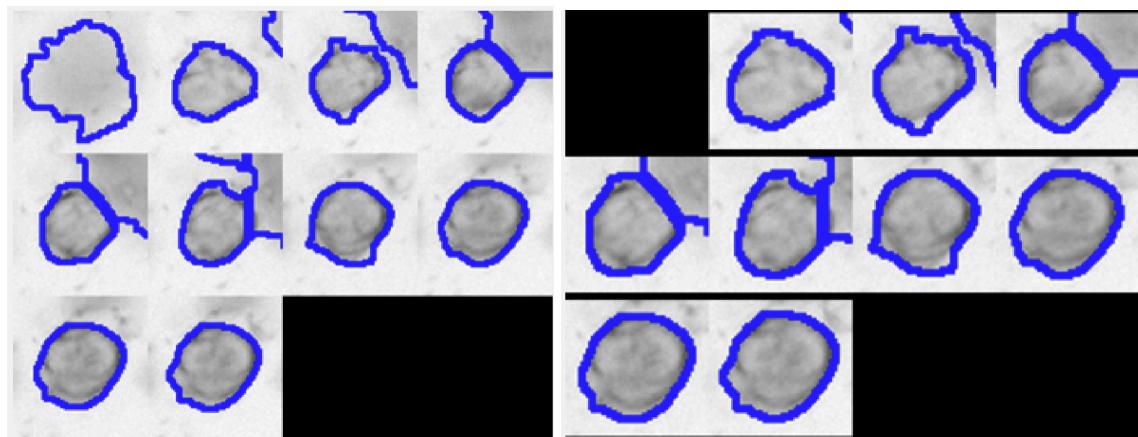
Registration	Sorting
Registration	Rigid (...
Num. Iterations [10-1000]	100
Degree of Smoothing [0.5 - 3]	1
Use Segmentation Mask	<input checked="" type="checkbox"/>
Use Raw Images	<input type="checkbox"/>
Num Workers	0
Register Batch	Register All
Done Registering	



5. Select “Done Registering”. Select “Track” to match segmented objects. Use the “Maximum Distance” edit field to adjust tracking sensitivity. A lower number requires objects to be closer in the registered image to be considered the same. The screen will display with each object given an ID, that matches organoids across images. Use the “ShowIDs” switch to turn labeling on and off for an image panel. Use the list box with numbers to select an ID and see the progression of that object across images. The list box only displays the information for the batch of organoids currently shown in the images.

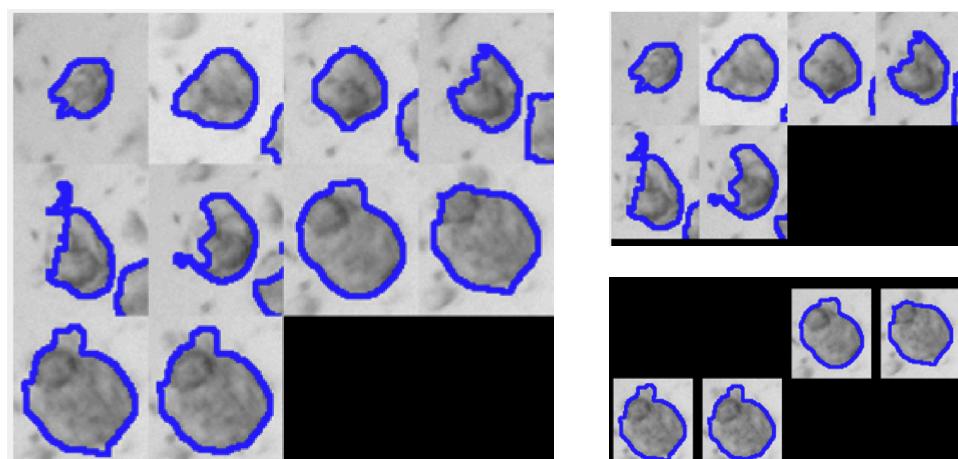


- a. Optionally, adjust the minimum/maximum growth parameters. By default, these are 0 and infinity, meaning size does not affect tracking. If these values are adjusted, organoids will not be matched to any organoid in the following image that does not fall in this growth range.



Min Growth = 0

Min Growth = 0.75



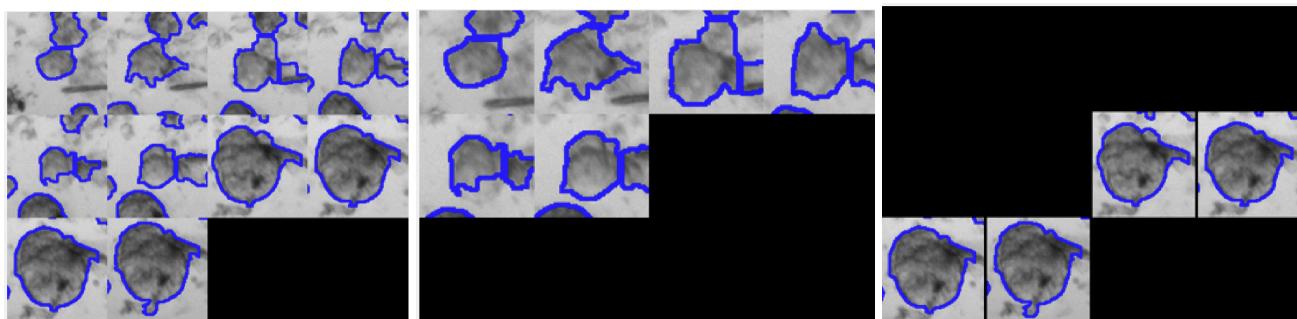
Max Growth = Inf

Min Growth = 2

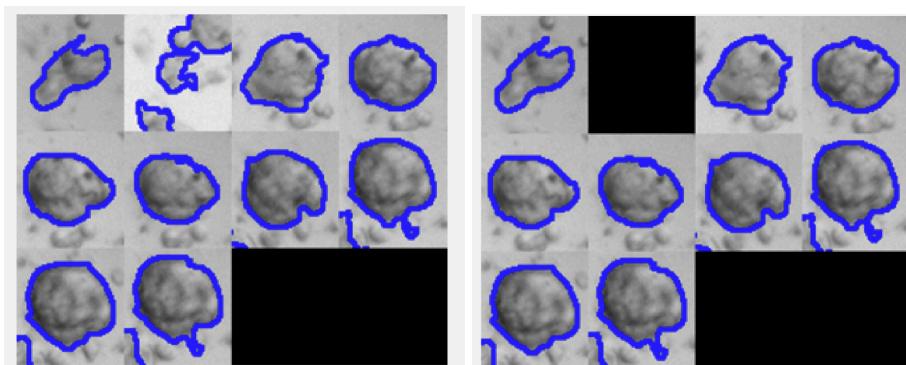
6. Use the options at the bottom to manually adjust tracking results if needed.
- a. If matching is too strict and a single organoid is separated into two IDs, select one of the organoid IDs in the list box. Use the “Spheroid #2” field to enter the ID of the other trace, and press “Match Spheroids”. This will fill in the empty entries of the current organoid with the entries from the second organoid at the respective time points.



- b. If matching is not strict enough and two distinct organoids are combined in one trace, select that organoid ID in the listbox. Then, enter the number of the first image where the incorrect assignment occurs into “First Wrong Image #”, and press “Unmatch Spheroids.” This will take all entries from that image and after, and turn them into a new trace with the next available ID.



- c. If a specific image or images in a trace are incorrect or poorly segmented, enter the number of the image (one at a time) in “Images to Remove,” and then press Remove from sequence. This will turn that image into its own organoid trace with the next available ID.



7. To manually verify individual organoid traces, use the up and down arrow keys to navigate between organoids. If a trace is incorrect, press “x”. This should add “_x” to the end of the organoid ID. In many cases, an organoid may be correctly tracked, but the segmentation is imperfect throughout. If you are seeking to analyze only exemplary organoid traces, press “c” on those traces, and “_c” should appear. Use the backspace to remove any designation. **You must click outside of the listbox to add designations to organoids**, as the GUI will not register keystrokes if the listbox (or any other UI component) is the last item you clicked on. You will still be able to use the up and down arrows to navigate through the list box, even after clicking outside of it.

8. Use the “Export Data” button to export metrics or a mat file. If “.xls” or “.xlsx” are selected, the app will export to Excel whatever metrics are selected from the main scene (either those from the Metric Selection scene, or all metrics if the switch is set). Each metric will be on a different sheet, with spheroid IDs listed vertically, and data from that spheroid in each image listed horizontally. The final sheet, labeled ‘ID in Original Segmentation’, shows the ID for each tracked organoid in the original segmented images in the main scene. If manual verification was performed, use the adjacent drop-down to determine which organoids are included in the Excel file (all organoids, only those marked with “c”, any that are not marked with “x”). Exporting to a .mat file will allow this window and all of the data to be opened again.

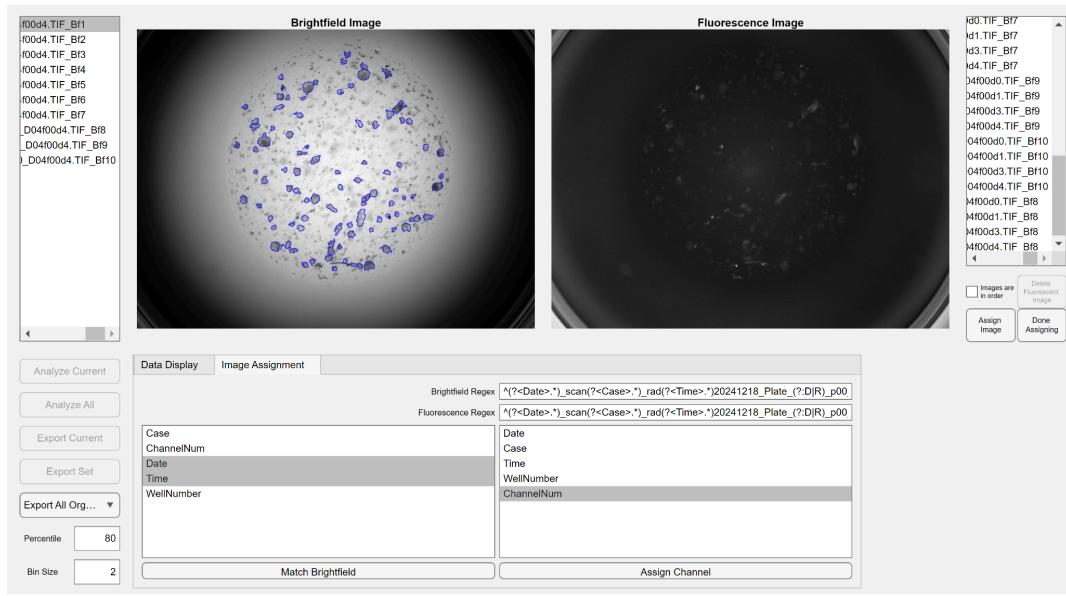
- a. If “Track In Batches” was used, a separate Excel or .mat file will be saved for each batch. When prompted for the file name to save, enter the base for how each file should be named, and then the app will append the grouping variables to the end of the filename (e.g., groupedTracking_149_C04.xls).
- b. To open the .mat file from the main OrganoSeg window, go to Additional Analysis → Tracking → Track Single Batch. Then, below the image list, select “Load Saved File,” and select the desired file. You will only be able to open one saved file per window.

Chapter 14: Fluorescence Analysis

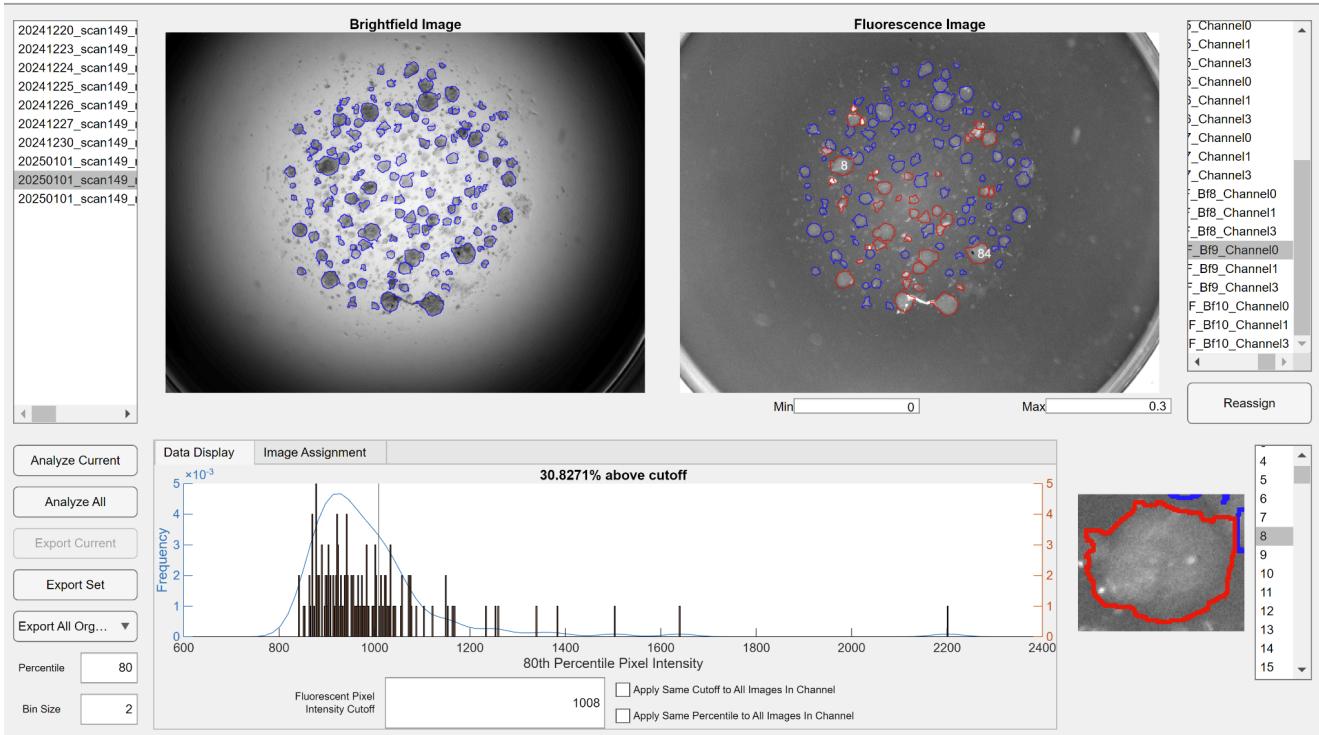
After segmentation of brightfield images, users have the option to perform fluorescence analysis given

images taken in fluorescent channels coregistered with the brightfield image.

1. Segment brightfield images and modify as desired
2. Select the fluorescence button (Additional Analysis → Fluorescence). Select for which brightfield image(s) you would like to analyze for fluorescence. Use Ctrl while clicking to select multiple images individually, and hold Shift and click on the last image in a range to select multiple sequential images.
3. Once on the fluorescence scene, go to File → Open Fluorescence Images or press Ctrl+o to open the desired fluorescent images. To delete an image, select it from the appropriate listbox and press “Delete Brightfield Image” or “Delete Fluorescence Images”. Once done, select “Done Opening Images”. You will **not** be able to add or delete images after this point.
4. Once all images have been opened, assign fluorescent images to the appropriate brightfield image (and channel if desired). If channels are not assigned, they will be automatically assigned based on image order. There are three options for the assignment. After applying the desired option, press “Done Assigning” to rearrange the images. Images will then be displayed as
“<ImageFileName>BF<CorrespondingBrightfieldImageNumber>_Channel<ChannelLabel>”
 - a. If the images are already ordered to correspond with the brightfield images, select the “Images are in order” check box. If you have more than one fluorescence image per brightfield image, you will need to choose how the images are ordered using Preferences → Images Grouping. The two options are “Image” or “Channel.” If your images are ordered so that all fluorescence images for the same brightfield image are together (e.g., day0_channel1, day0_channel2, day1_channel1, day1_channel2), use “Image”. If they are ordered so that all images of the same channel are together (e.g., day0_channel1, day1_channel1, day0_channel2, day2_channel2), use “Channel.” Once the checkbox is selected, the fluorescence image names in the listbox will be appended with “_BF#”, where # corresponds to the matched brightfield image. If the number of fluorescence images is not an exact multiple of the number of brightfield images, automatic assignment may not work as desired. You can reassign selected images as described in Option c.
 - b. To apply matching and channel assignment based on filenames, use a regex similar to that described in Chapter 4. In the “Image Assignment” tab at the bottom, you may assign separate regex strings (or copy the same one) for brightfield and fluorescence images. The left listbox is for matching fluorescence images to brightfield and will populate with all key variables that appear in both brightfield and fluorescence regex strings. The right listbox is for assigning channels, and will populate with all variables in the fluorescence regex string. For each, select one or more variables, and then press the appropriate button. Once pressed, the fluorescence image names in the listbox will be appended with either “_BF#” or “ChannelX”, where # corresponds to the matched brightfield image, and X corresponds to a label determined based on the select variable.
 - c. The final option is to manually assign images. Select a fluorescence image and the brightfield image to be assigned, and then press “Assign Image.” Manual assignment must be done for each fluorescence image.



5. Select “Analyze Current” or “Analyze All” to analyze the current image pair or all image pairs. This will overlay the fluorescent image with the brightfield segmentation and display a histogram of representative pixel intensities for each spheroid.
6. Go to the “Data Display” tab at the bottom to visualize a histogram and smoothed density function of the fluorescence intensity distribution. Organoids that are above the cutoff will be highlighted in red.



7. (Optional) Use the “Min” and “Max” edit fields below the fluorescence image to scale image intensity relative to the true min and max values. This only affects display and has no impact on analysis.

8. (Optional) Choose different colors to outline spheroids (either all spheroids or fluorescently marked spheroids), depending on the color of the fluorescence or personal preference. (Preferences → Perimeter Colors → All spheroids or Fluorescently marked). A prompt will ask whether you want the new outline to apply to the current image, all images in the current channel, or all images.

9. Adjust parameters to determine which spheroids are marked.

Percentile (default: 80): This determines what percentile of pixel intensity summarizes the fluorescence intensity of each spheroid. Choosing 100 means that each spheroid will be summarized by its brightest pixel in the fluorescent image. Choose “Analyze Current” or “Analyze All” after changing the percentile to update the display.

- **Apply Same Percentile to All Images in Channel:** Select this checkbox to use the same pixel intensity cutoff for all images, which will automatically update the pixel intensity for other images. To have the same percentile for all images, apply this change once to each channel.

Bin Size (default: 5): This changes the size of bins displayed in the histogram, which will automatically repopulate after the change. Bin size has no effect on analysis results, but it may affect how the distribution is perceived.

Fluorescent Pixel Intensity Cutoff. Any spheroid whose summarized pixel intensity is equal to or greater than this value will be marked as fluorescent. This value can be chosen by entering a value into the edit field or clicking on the histogram at the appropriate value. The current value is indicated with the vertical line on the histogram. Changing the cutoff value regenerates the fluorescent display.

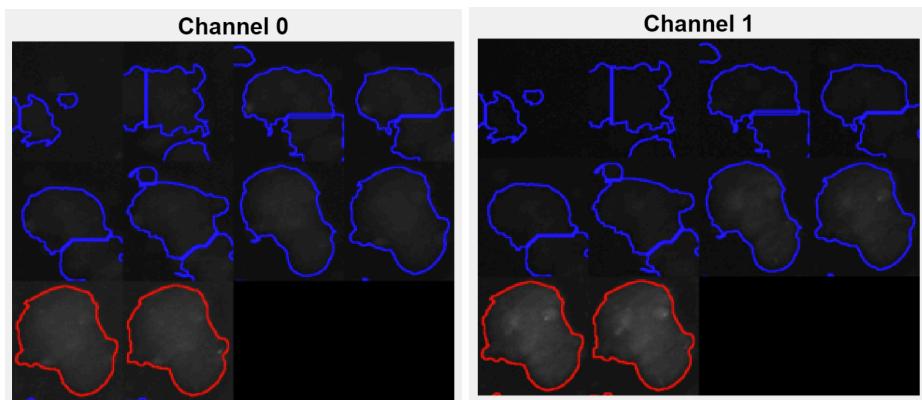
- *Apply Same Cutoff to All Images in Channel:* Select this checkbox to use the same pixel intensity cutoff for all images in the current channel, which will automatically update the pixel intensity for other images. To have the same cutoff for all images, apply this change once to each channel.

10. Export data using “Export Current” or “Export Set”. Data are exported to an Excel file containing the representative pixel intensity, whether the spheroid was fluorescently marked or not, and the area.

Fluorescence Analysis of Tracked Organoids:

After tracking organoids, users can perform fluorescence analysis on tracked organoids and monitor the change in fluorescence for each organoid.

1. Select the Fluorescence option in the menu to open the fluorescence scene. If “Track in Batches” was selected, only the batch of images that are currently displayed will be loaded. Fluorescence analysis of tracked organoids is only available for one batch at a time.
2. Use the same process as described above to open and assign images. **Channel selection is critical for fluorescence analysis using tracked organoids**, as this determines what data will be longitudinally grouped together. If a regex string is not used for channel assignment, channels will be assigned in order for all fluorescent images with the same brightfield image. Do not use automated assignments if certain channels are not present at every time point.
3. Proceed with fluorescent analysis as described at the beginning of Chapter [14](#).
 - a. Spheroid IDs will be displayed as the matching ID assigned during tracking.
 - b. You will have the option to “See montage”. Selecting this option will replace the spheroid zoom with a montage of the selected spheroid in each image of a given channel. Users may change the channel shown by selecting the next or previous channel buttons.
 - c. Image intensities are scaled on a per-channel basis, so that spheroid montages are compared side-by-side on the same scale.



- d. Exporting produces an Excel document where each sheet represents one of the three fluorescence metrics for a given channel, and the data display the values for these metrics across each image in the time series for each organoid.

Chapter 15: Keyboard Commands

If keyboard commands are not working, the user may need to click anywhere on the screen that is not an app component (button, edit field, checkbox, etc.) and try the keyboard command again.

Main scene:

- ‘ctrl’/‘control’ or ‘command’ + ‘o’ - open new images
- ‘s’ - Segment current image
- up/down arrow keys - display previous/next image
- ‘e’ - escape tools and disabled buttons
- ‘enter’/‘return’ - confirm tool selections (crop, remove, split, correct, change classification)
- ‘z’ - undo
- ‘r’ - select remove tool
- ‘c’ - select combine tool
- ‘t’ - select individual splitting tool
- ‘i’ - select individual edge correction tool
- ‘1’ - select “make spheroid” classification tool
- ‘2’ - select “make contaminant” classification tool
- ‘3’ - select “make non-circular” classification tool
- ‘4’ - remove all contaminant items in bulk
- ‘5’ - remove all non-circular items in bulk
- ‘x’ - select crop tool

Tracking scene:

- ‘enter’/‘return’ (in tracking analysis scene) - confirm selection for manual registration
- up/down arrow keys - display previous/next organoid
- ‘x’ - mark as bad organoid trace
- ‘c’ - mark as exemplary organoid trace
- ‘backspace’ - remove organoid designation

Chapter 16: Currently Known Bugs

- Manual reclassification (for contaminants or non-circular objects) will reset if combining, edge correction, or reverting are performed and the respective parameter (minimum circularity or contaminant intensity) is non-zero, given the need to recalculate circularity or pixel intensity for modified spheroids. However, if manual reclassifications are made and then bulk removal is performed, the changes will persist.
- Certain actions, such as segmenting and exporting, will disable GUI buttons to prevent repeated callbacks and indicate that the action was registered. If an error occurs during the execution of these actions, the buttons will remain disabled. In the case that this occurs, press ‘e’ to escape and enable buttons.
- Exiting out of a waitbar will not interrupt the completion of an action.