

MiA User Guide

Alexis Pasulka, Jonathan Hood

Cal Poly, San Luis Obispo ©2022

Introduction

The Microbial Image Analysis (MiA) program was designed specifically for use in analyzing epifluorescence microscopy images, but may prove useful in other applications. The program is compatible with grayscale and RGB images in a .tiff format, as well as those in a CZI format (e.g., Zeiss images). The program load any number of channels for a given image. After defining regions of interest (ROIs), the data can be exported as a .txt, .csv, or .xlsx file. Several alternate saving options exist, discussed in detail in Section 4.1.

Contents

1 System Requirements	4
2 Installation Procedure	4
2.1 MATLAB Program Version	4
2.2 Executable Program Version	4
3 Detailed Guide to Workflow	5
3.1 Opening the Program	5
3.1.1 Opening from the Executable	5
3.1.2 Opening from the Script in MATLAB	6
3.1.3 Initial Program Interface	6
3.2 Loading an Image	6
3.3 Changing Image Properties	8
3.4 Changing Output Directory	8
3.5 Selecting Threshold Channel, Connectivity, & other Displayed Properties	9
3.6 Defining Your Regions of Interest (ROIs)	9
3.6.1 ROI Statistics	11
3.6.2 Undo and Redo ROI	11
3.6.3 Autosaving	12

3.7	ROI Identification	12
3.8	Saving Data, Masks, & Images	12
4	Explanation of Menu Functions	13
4.1	File Menu	13
4.1.1	Load Image	13
4.1.2	Saving Options	14
4.1.2.1	Save ROI Data & Masks	14
4.1.2.2	Save ROI Data	17
4.1.2.3	Save Images Only	17
4.1.2.4	Save ROI Mask	17
4.1.2.5	Save Outlined Cells Image	17
4.1.2.6	Save ROI IDs	17
4.1.3	Undo ROI	19
4.1.4	Redo ROI	19
4.1.5	Export As	19
4.1.6	Exit	19
4.2	ROI Tools Menu	19
4.2.1	Threshold Channel	19
4.2.2	Connectivity	20
4.2.3	Background Subtraction	20
4.2.4	Auto Threshold ROI	22
4.2.5	Manual Threshold ROI	22
4.2.6	Auto Threshold Region of ROIs	27
4.2.7	Manual Threshold Region of ROIs	27
4.2.8	Manual Threshold All ROIs	30
4.2.9	Draw Ellipse ROI	32
4.2.10	Draw Freehand ROI	33
4.2.11	Split ROI	35
4.2.12	Delete ROI	36
4.2.13	Delete Region of ROIs	36
4.2.14	Delete All ROIs	38
4.2.15	Load Mask	38
4.2.16	Load Autosaved Mask	38
4.2.17	Identify ROIs	38

4.3	Display Menu	40
4.3.1	Zoom	40
4.3.2	Default View	41
4.3.3	Reset Channel Color Contrasts	41
4.4	ROI Statistics Panel	42
4.5	Image and Output Directory Filepaths	43
4.6	Image Options Panel	43
4.6.1	Displayed Properties Tab	43
4.6.2	Channel Properties Tab	44
4.6.3	Image Properties Tab	46
5	Licensing	47

I System Requirements

The MiA program can either be run as a script inside the MATLAB software (Option 1) or as an executable outside of the MATLAB software (Option 2).

Option 1) In order to run the program in MATLAB from the script, you will need to install:

1. MATLAB Version 2020a or later.
2. MATLAB Image Processing Toolbox.
3. MATLAB Computer Vision Toolbox.
4. Platform-specific MiA program files.

Option 2) In order to run the program as an executable program outside of the MATLAB software, you will need to install:

1. MATLAB Runtime Environment (latest version).
2. Platform-specific MiA executable program.

2 Installation Procedure

2.1 MATLAB Program Version

Download the MiA program files appropriate for your system (Mac or Windows). You can follow the instructions for opening the script in MATLAB in Section 3.1.2.

2.2 Executable Program Version

Download the MiA executable program appropriate to your system (Mac or Windows). Once you've downloaded the executable installer, you will need to navigate to its location on your computer and open it. A pop-up may appear verifying the download with publisher 'Unknown'. Follow the instructions of the program, including selecting an installation location. Once you do so and accept the Mathworks licensing agreement, the download will begin. (*NOTE: The program will not download the runtime environment if it detects it has already been downloaded*).

- If needed, you can download the runtime environment here.

To Run the program after installation, navigate to the folder where you installed the program and open the application per Section 3.1.1.

*****Mac Users***** *There is an issue with some Mac operating systems that prevents the MiA installer from opening due to developer permission issues. You may receive a pop-up message that says "macOS can not verify the developer of MiA Installer. Are you sure you want to open it?". In some cases, simply clicking "open" will enable the program to run. In other cases, you will need to do the following: Open System Preferences. Click*

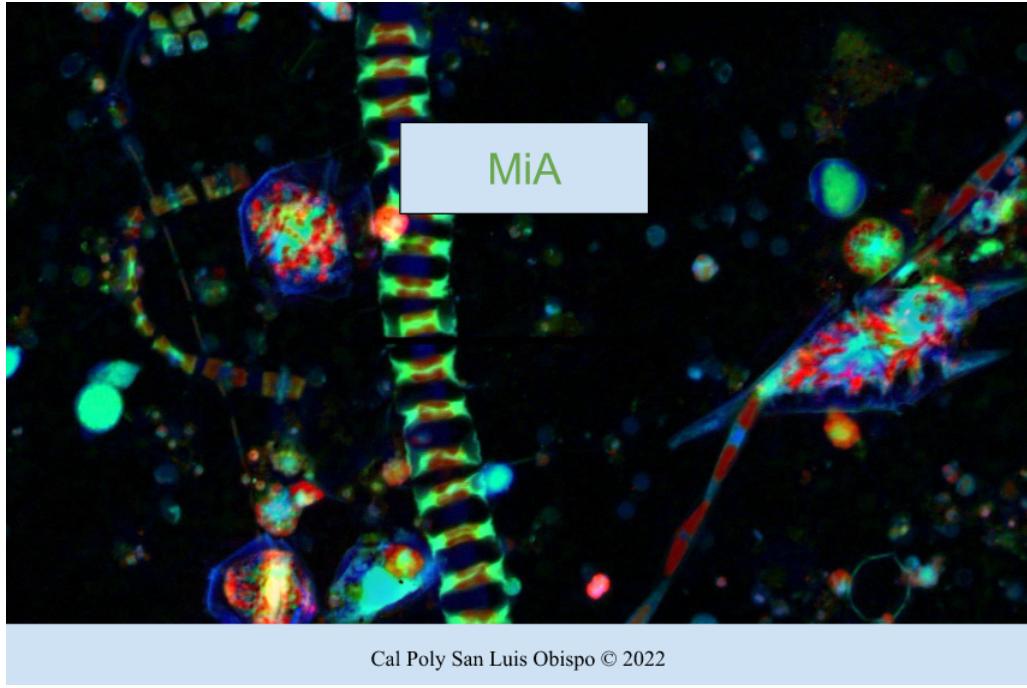


Figure 1: Windows MiA Splash Screen, 2022 edition

General, then click on the lock button in the bottom left-hand corner, as indicated above by the yellow arrow. You will be prompted to fill in the password you use to unlock your computer. Once you have done so, you will be able to click “Open Anyway” next to the message “MiA Installer was blocked from use because it is not from an identified developer”.

Please also note that there is an incompatibility between window-snapping apps (e.g., Magnet App, BetterTouchTool) and MATLAB on Mac OS. This incompatibility persists even when using the MATLAB Runtime Environment and these programs must be closed in order to use this MATLAB-based program.

3 Detailed Guide to Workflow

This section of the manual details the full typical workflow, from loading an image to exporting data. For a detailed explanation of the individual menu options, see Section 4.

3.1 Opening the Program

3.1.1 Opening from the Executable

Assuming the executable is installed on your machine locally, you can run it just like any other computer program. It will be located within the ‘application’ sub-folder of the folder you selected for installation. The program will either be an .exe or .app file depending if you installed it onto a PC or Mac, respectively. Double-click the .exe (or .app) file and the program will start up. The executable version of the program may be slow to open, but it will then have the same functionality as the MATLAB version. You should be greeted by a splash screen similar to Figure 1:

3.1.2 Opening from the Script in MATLAB

Open MATLAB and navigate to the proper working directory (e.g., the folder you saved the script files in). It is important to note that finding the proper working directory will differ slightly depending on whether you're using a Mac or Windows OS.

The scripts to run the program are organized into a series of MATLAB packages. They can be distinguished by the '+' present in every folder's name. The primary file to run is located in the '+Interfaces' package and is labelled 'image_analysis.m'.

If you're using a Windows OS, make sure you're in the directory just above the '+Interfaces' package itself; in other words, you should be able to see all of the packages in your MATLAB working directory. MATLAB greys out files and folders that are not in your current working directory, so as long as the 'image_analysis.m' file isn't greyed the program should run.

If you're using a Mac OS, the working directory will be slightly different. You'll need to go inside the package itself before running the program. An example directory view is shown in Figure 2.

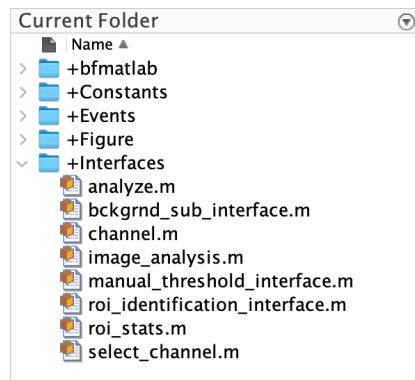


Figure 2: Working Directory

To run the program, you can either open up the 'image_analysis' file in the command window and click 'Run'. This will run the code as a regular MATLAB file. Or you can run it from MATLAB's command window by calling the script directly, and the program will run.

3.1.3 Initial Program Interface

Figure 3 displays the initial interface presented to the user upon opening the program.

The interface is divided into four primary sections: 1) the leftmost panel, which holds the statistics of the identified regions of interest; 2) the center-top panel, which displays the loaded image and axes upon which the user can identify ROIs; 3) the center-bottom panel, which holds the filepaths to the loaded image and to the selected output directory; and 4) the rightmost panel, which holds the displayed image properties in the first tab, and the individual properties of each channel in the second tab.

3.2 Loading an Image

There are two ways to load an image: 1) From the 'File' menu, using the 'Load Image' sub-menu as detailed in Section 4.1.1, or 2) Using the '...' push button located directly to the right of the 'File Select...' edit box near the bottom of the application window (Figure 3).

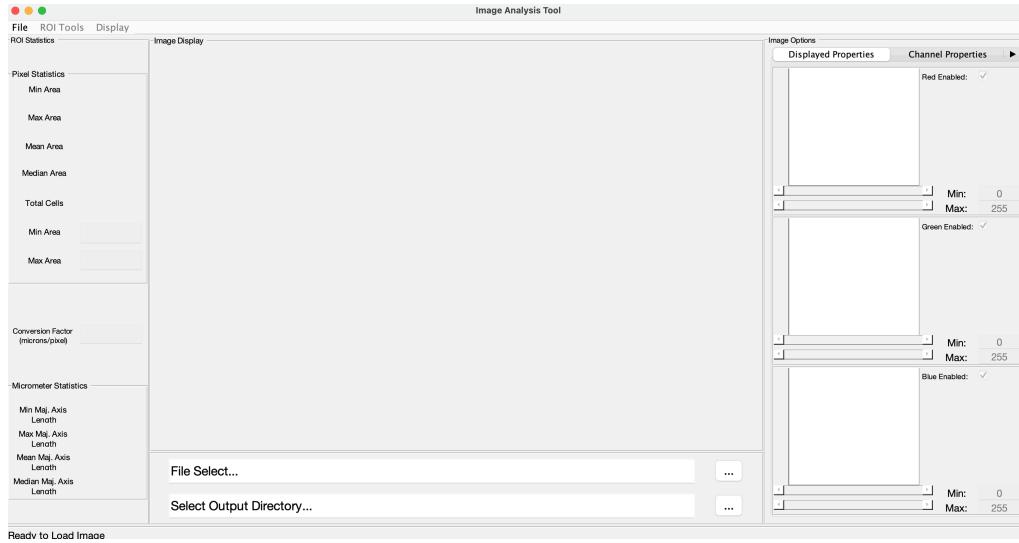


Figure 3: Initial Image Analysis Interface

Either of these options brings up a file explorer option native to your operating system. You can load three different types of images including CZIs (Zeiss proprietary image type), RGBs, or Grayscale images. You can load only one CZI or RGB image at a time; however, you can load as many grayscale images as you want during this initial selection.

For RGB Images

- The image will load automatically into the main window.

For CZI and Grayscale Images

- A small selection dialog will pop up, allowing you to assign loaded channels to the three available color channels (Red, Green, and Blue) visualized in Figure 4. Only three channels can be displayed at any given time, but all CZI channels/grayscale images are stored and can be swapped out for loaded channels in the 'Channel Properties' tab.

Once loaded, image information is displayed in the 'Image Properties' tab of the 'Image Options' panel on the rightmost side. You can use the **Changing Image Properties** to make any adjustments after loading.

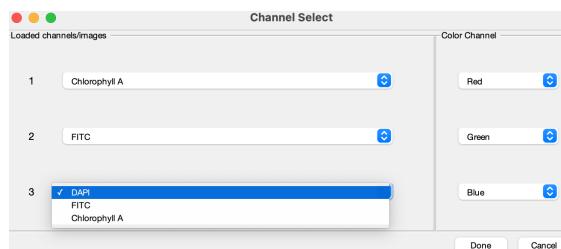


Figure 4: Channel Select Pop-up

******WARNING****:** There is currently an error in Mac OS (Catalina or later) communications to MATLAB resulting in an inability to load anything other than the first filter index, in this case, CZI images. This issue has been resolved by reverting to a native Java file chooser; however, that functionality may be removed by MATLAB in a future release in which case program updates will be required.

3.3 Changing Image Properties

After loading an image, depending on what information is detected, the 'Channel Properties' tab will populate, looking similar to Figure 5. In this tab, you can change the names of individual channels, swap which channels are displayed as each color, and input/edit each channel's individual exposure time. Everything changed in this tab will affect what and how data is exported; for instance, if exposure time is added, the exposure time of that channel will be included in the exported data sheet. Most loaded CZIs will populate exposure time directly if it is detected; for .tiffs, you will need to manually input exposure time directly, if known.

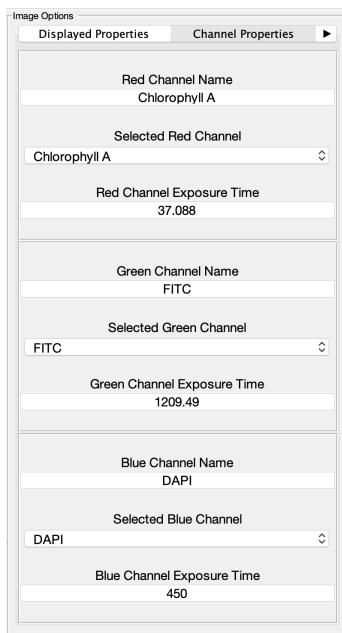


Figure 5: Example Channel Properties Tab

3.4 Changing Output Directory

The output directory is where all saved data, images, and masks will be placed. You can either enter the output directory in the editable text box as shown in Figure 6, or you can navigate to a directory by selecting the '...' button directly to the right of the editable box. This directory can either be set before image selection or after. If no output directory is selected upon loading an image, the output directory defaults to the directory the image was loaded from.

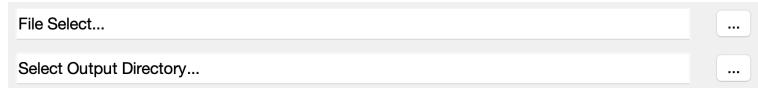


Figure 6: File-Directory Selection Snapshot

3.5 Selecting Threshold Channel, Connectivity, & other Displayed Properties

The most common steps before analysis typically include 1) choosing a threshold channel, 2) setting a connectivity, and 3) altering other displayed properties.

1. The threshold channel is defined as the color channel the program will use to determine the threshold level for the automatic and manual thresholding techniques. If most of the image's desired ROIs appear to be in the blue channel for instance, it might be advantageous to threshold based only on the blue channel.
2. Connectivity defines which pixels are connected to other pixels when an ROI is selected. A more detailed description is available in Section 4.2.2.
3. In addition to the options that directly affect programmatic analyses, there are several options to better assist in manual ROI definition, such as disabling channels and editing contrasts.
 - (a) The 'Displayed Properties' tab of the 'Image Options' panel, as displayed in Figure 7, allows the manipulation of individual channel contrasts and the disabling/enabling of color channels. By manipulating the sliders or specifying the minimum and maximum for each channel, the contrast of the selected channel is normalized to the new values. This can be very useful in bringing faint ROIs to the forefront. In addition, each color channel section has an 'Enabled' checkbox. When checked, the color channel is displayed; when unchecked, the color channel disappears from view. **It's important to note that changes made in the displayed properties window do not alter the actual data and only influence real-time visualization while analyzing the image. When the data is exported, any changes made in this panel are not reflected in the data.** However, changes made in the channel properties window do influence the data.
 - (b) The 'Display' menu in the primary toolbar also contains a few options to assist in harder-to-define ROIs or shortcuts to reset views. The user can zoom in on sections of the image as needed using either the 'Zoom' option in the 'Display' menu drop-down or using the hotkey 'Ctrl+Z'.
 - (c) The user can also reset channel contrast adjustments here, as well as reset the zoom level to default view.

3.6 Defining Your Regions of Interest (ROIs)

The next step in image analysis is to identify your regions of interest (ROIs). There are a range of tools available to define your ROIs (Table 1). You can complete the selection of your ROIs in one session or you can save a mask (see Section 4.1.2.4) and come back and complete ROI selection in a later session.

NOTE: If you've already created ROIs and wanted to load a previously created mask, you can do that once you've loaded an image (see Section 4.2.15 for more information).

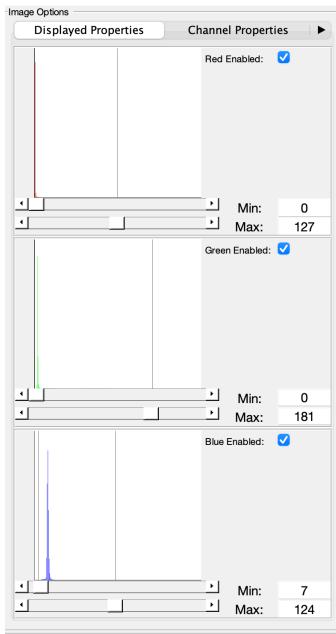


Figure 7: Contrast/Channel Display Panel

Table 1: Available ROI Tools

ROI Tools	Description
Automatic Threshold ROI	Lets you draw a freehand region on the image. By double-clicking and finalizing the freehand, the program auto-thresholds the region and defines the largest ROI it discovers. For more information, see Section 4.2.4.
Manual Threshold ROI	Lets you draw a freehand region on the image. By double-clicking and finalizing the freehand, the manual threshold tool appears and lets you define the threshold for that region. Only the largest ROI is defined. For more information see Section 4.2.5.
Auto Threshold a Region of ROIs	Lets you draw a freehand region on the image. By double-clicking and finalizing the freehand, the program auto-thresholds the region and defines all ROIs it discovers. For more information, see Section 4.2.6.
Manual Threshold a Region of ROIs	Lets you draw a freehand region on the image. By double-clicking and finalizing the freehand, the manual threshold tool appears and lets you define the threshold for the region. All detected ROIs are kept. For more information, see Section 4.2.7.
Manual Threshold All ROIs	The manual threshold tool appears and lets you define a threshold for the entire image. All detected ROIs are kept. For more information, see Section 4.2.8.

Table 1: Available ROI Tools

ROI Tools	Description
Draw Ellipse ROI	Lets you define an ellipsoid region on the image. It can be dragged, rotated, prolated and oblated until it best matches the ROI you'd like to define. Once defined, double-click to finalize. See Section 4.2.9 for more information.
Draw Freehand ROI	Lets you define a freehand region on the image. It can be defined in any direction using waypoints until it best matches the ROI you'd like to define. Once defined, double-click to finalize. See Section 4.2.10 for more information.
Split ROI	Lets you split one or multiple ROIs into separate ROIs. Draw a single line freehand between two or more ROIs. The line can be of any path shape; all ROIs intersecting the line will be split along it. Occasionally, this feature will not work properly; if so, 'Undo ROI' as described in Section 3.6.2 and try again. See Section 4.2.11 for more information.
Delete ROI	Lets you select a single ROI to remove. See Section 4.2.12.
Delete Region of ROIs	Lets you draw a freehand region surrounding multiple ROIs. Once you double-click to finalize the region, all ROIs within will be deleted. See Section 4.2.13 for more information.
Delete All ROIs	After asking for confirmation, removes all ROIs in an image. See Section 4.2.14 for more information.

3.6.1 ROI Statistics

After defining a few ROIs (or loading a mask) the ROI statistics panel will populate with information. In this panel, you'll get a snapshot view of the general stats of the ROIs defined so far, including the min, max, mean, and median ROI pixel area, as well as the total number of cells. This is also the place where you can filter the ROIs by area, either by minimum or maximum. Additionally, if the conversion factor for the microscope from microns to pixels is known, it can be filled in here, and major axis length data in terms of microns will be populated. Refer to Section 4.4 for more information.

3.6.2 Undo and Redo ROI

With some ROIs defined and more on the way, you might find yourself accidentally deleting a useful ROI or creating several unwanted ones. This can be easily undone, or redone, with the 'Undo' and 'Redo' ROI options in the File menu. More information on these options can be found in Section 4.1.3 and Section 4.1.4.

3.6.3 Autosaving

The binary mask (e.g., the file that contains the outlines of your ROIs) will autosave every time a change is made to the mask. Therefore, if the program crashes for any reason, you can load the autosaved mask directly, either by the option 'Load Autosaved Mask' or just 'Load Mask'. For more info on how those work, check out Section 4.2.16 and Section 4.2.15. See more details below in Section 3.8 for more information on saving masks and your data.

3.7 ROI Identification

The program provides an option for manually identifying ROIs as particular taxa or groups. Navigate to 'Identify ROIs' under the 'ROI Tools' menu options. This will open up a new window with a zoomed in view of the first ROI and several options for assigning an ID, as well as zooming options, and different ways to swap between which ROI is being viewed. These designations can be saved when exporting the data. For more details, see Section 4.2.17.

3.8 Saving Data, Masks, & Images

Once you're done defining ROIs, and possibly identifying them, it's time to save your data. There are several different save options, briefly listed in Table 2 below.

Table 2: Available Save Options

Save Option	Description
Save ROI Data & Masks	Exports ROI data based on current binary image mask. A full explanation of what data is exported is listed in Section 4.1.2.2. In addition, exports the current binary mask under the image name or a user-defined name. More detail for that operation can be seen in Section 4.1.2.4.
Save ROI Data	Exports ROI data based on current binary image mask. A full explanation of what data is exported is listed in Section 4.1.2.2.
Save Images Only	Saves three snapshots related to current image; numbered cells, outlined cells, and the regular image. The images are exported with the current contrasts and/or channel selections for visualization purposes only. For more detail, see Section 4.1.2.3.
Save ROI Mask	Exports the current binary mask under the image name or a user-defined name. More detail for that operation can be seen in Section 4.1.2.4.
Save Outlined Cells Image	Saves only the 'Outlined Cells' image, the current setup including contrast adjustments including ROI outlines.
Save ROI IDs	Saves all ROI IDs as a .csv file, assuming any have been defined. For more details see Section 4.1.2.6.

It's important to note that saving data and masks are two different things. The ROI 'data' refers to the statistical data taken from ROIs, while the ROI 'mask' is the actual binary mask that MATLAB uses to define the location of the ROIs on the image. Once exported, you can work with the ROI data (in a .csv or .xlsx) in other programs (MATLAB, R). However, the ROI masks are valuable as these files are small and can be reloaded onto the image at any point for future analyses. In addition to these save options, you can save other images to acquire a quick snap shot of your current analysis including the outlines or ROI numbers. These snapshot images maintain the current channel and threshold settings of your session, but do not influence the raw data or original image.

4 Explanation of Menu Functions

This section of the manual details the function of each individual selection. For a workflow example, see Section 3 above.

4.1 File Menu

The file menu is located at the top-left corner of the initial image analysis interface. Each available option in this menu will be explained in depth below; however, the brief operative process is to load an image using 'Load Image(s)', and, after marking ROIs and editing channel properties, employ one of the many 'Save' options (Table 2) to export the data.

4.1.1 Load Image

'Load Image(s)' enables the user to load one or multiple images. Currently, three image types are supported for analysis:

1. RGB images in .tiff formats.
2. Grayscale images .tiff formats.
3. CZI images in .czi format.

The default option during image selection is CZI, as shown in Figure 8.

The file extension can be changed to either RGB or grayscale to display .tiff image options instead. Ensure that the correct extension, RGB or grayscale, is selected in the file extension dropdown before loading the image. With the grayscale file extension selected, the user can select any number of .tiff images. However, when either RGB and CZI images are selected, the user will only be able to load one image. Loading multiple images with a file extension other than grayscale selected will result in the program asking you to load a single non-grayscale image.

The program is capable of loading a CZI image with any number of channels; however, for simplicity, only three channels can be displayed/edited at any given time. As described in Section 4.6.2, it is possible to swap out the non-displayed CZI channels with the displayed CZI channels at the user's discretion. For the special export options resulting from detected but not displayed channels, see Section 4.1.2.2, 'Save ROI Data'.

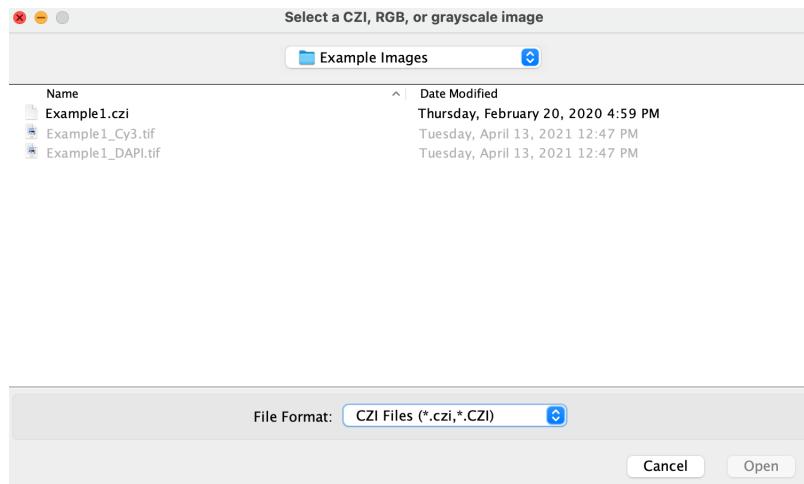


Figure 8: Example 'Load Image(s)' Dialog

If less than three images (or less than 3 channels within a CZI) are loaded, the remaining color channel(s) will be populated with blank, or zero-image, channels. These blank color channels have identical properties to loaded color channels, allowing name change and exposure time setting, but, similar to the 'None' channel described in Section 4.6.2, will not be exported or included in any statistical data.

When multiple grayscale images or a single CZI image with multiple channels is loaded, another 'Channel Selection' dialog will appear, allowing the user to assign color channels to either each loaded grayscale image/populated blank image, or each loaded CZI channel. This step can be visualized in Figure 9.

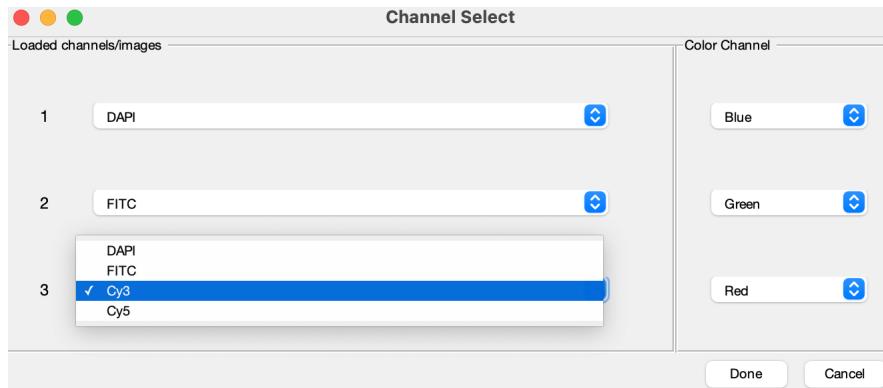


Figure 9: Example 'Channel Select' Dialog: Four-Channel CZI

4.1.2 Saving Options

4.1.2.1 Save ROI Data & Masks

The 'Save ROI Data & Masks' option enables the user to save two key elements of image analysis and is recommended as the default save option. The ROI data exports data based on the current binary image mask. The ROI data will be exported to the selected output directory in the file format checked

under 'Export ROI Data As...'. The ROI mask gets saved as the current existing ROI binary mask under either the image name (default) or a user-defined name in the selected output directory. Once the mask name has been selected, the user will not be prompted again for the duration of the session. If any ROIs have been identified as described in Section 4.2.17, the ROI IDs are exported with the data as well as saved in the created .mat file associated with the masks. For information on reloading a previously saved mask, refer to Section 4.2.15.

Below is the information that gets saved in the ROI data file for each image when exporting the data, where NAME indicates the name of each channel and COLOR indicates the color channel that image channel was assigned to:

1. **image_name**: the name of the image itself, e.g. 'plankton_image.czi'
2. **roi_num**: the individual ROI ID number, e.g. '1, 2, 3, 4, ...'
3. **roi_id**: if any ROIs have been identified, there will be a column here between 'roi_num' and 'COLOR_NAME_max' with each ROI's individual string identifier
4. **COLOR_NAME_max**: the maximum intensity pixel of channel NAME within the ROI's area. Assigning channel 'DAPI' to the 'Blue' channel will result in the name 'b_dapi_max', while assigning channel 'FITC' to the 'Red' channel will result in the name 'r_fitc_max'.
5. **COLOR_NAME_min**: the minimum intensity pixel of channel NAME within the ROI's area. Assigning channel 'DAPI' to the 'Blue' channel will result in the name 'b_dapi_min', while assigning channel 'FITC' to the 'Red' channel will result in the name 'r_fitc_min'.
6. **COLOR_NAME_mean**: the mean intensity pixel of channel NAME within the ROI's area. Assigning channel 'DAPI' to the 'Blue' channel will result in the name 'b_dapi_mean', while assigning channel 'FITC' to the 'Red' channel will result in the name 'r_fitc_mean'.
7. (OPTIONAL) **COLOR_NAME_exp**: If the channel was loaded with or manually given an exposure time, this data column will display the exposure time of channel NAME in seconds. Assigning channel 'DAPI' to the 'Blue' channel will result in the name 'b_dapi_exp', while assigning channel 'FITC' to the 'Red' channel will result in the name 'r_fitc_exp'. If no exposure time was found or assigned to a channel, this column will not export for that channel.
8. (OPTIONAL) **COLOR_NAME_background_subtracted_max**: If background subtraction operations were performed on the channel NAME, this data column will display the maximum intensity within a given ROI after background subtraction operation. This column only appears if background subtraction operations were performed on channel NAME.
9. (OPTIONAL) **COLOR_NAME_background_subtracted_min**: If background subtraction operations were performed on the channel NAME, this data column will display the minimum intensity within a given ROI after background subtraction operation. This column only appears if background subtraction operations were performed on channel NAME.
10. (OPTIONAL) **COLOR_NAME_background_subtracted_mean**: If background subtraction operations were performed on the channel NAME, this data column will display the mean intensity of a given ROI after background subtraction operation. This column only appears if background subtraction operations were performed on channel NAME.
11. **area**: the area (actual number of pixels in the region) of the ROI

12. **centroid_1**: the x-coordinate in pixels of the centroid of the ROI, relative to an origin located at the bottom-left of the image.
13. **centroid_2**: the y-coordinate in pixels of the centroid of the ROI, relative to an origin located at the bottom-left of the image.
14. **major_length**: the length of the major axis of the ROI, as approximated by an ellipse, in pixels.
15. **minor_length**: the length of the minor axis of the ROI, as approximated as an ellipse, in pixels.
16. **perimeter**: the perimeter of the ROI in pixels.

Summary Data - When saving data as an Excel workbook, a 'Data Summary' sheet also gets saved as a second sheet in the file. This sheet contains the data visible in the 'ROI Statistics' panel of the primary program interface including total cells and min/max/mean/median ROI area in pixels. If a pixel to micron conversion factor was included, min/max/mean/median ROI major axis length in micrometers and min/max/mean/median ROI area in micrometers are also displayed. If background selection was performed, the 'rolling ball' algorithm shape that was selected and its size in pixels is displayed.

An example data output (Excel-type) is as displayed in Tables 3, 4 and 5. Note that the first row of each table represents a single real-data row of headers, while the second row of each table represents a single real-data row of data.

*NOTE: Exported ROI pixel data is in the numerical units of the **original, unedited** channel data. For most CZIs, this unit is 'uint16', which results in intensities in the range of hundreds to tens of thousands.*

Table 3: Example Output Data, Columns 1–6

image_name	roi	b_dapi_max	b_dapi_min	b_dapi_mean	b_dapi_background_subtracted_max
plankton_image.czi	1	8204	695	1368.77	7204

Table 4: Example Output Data, Columns 7–9

b_dapi_background_subtracted_min	b_dapi_background_subtracted_mean	b_dapi_exp
595	1000	228.658

Table 5: Example Output Data, Columns 10–15

area	centroid_1	centroid_2	major_length	minor_length	perimeter
3222	31.68	165.7	85.26	51.27	358.381

NOTE: These tables include only one exported channel, a 'DAPI' channel exported as 'Blue'. Any additional exported channels would repeat as Columns 3–9, between Columns 9–10. For instance, if a 'FITC' channel was exported as 'Green, 'r_dapi_exp' in Column 9 would be followed immediately in Columns 10–16 by 'g_fitc_max', 'g_fitc_min', 'g_fitc_mean', g_fitc_max_background_subtracted, g_fitc_min_background_subtracted, g_fitc_mean_background_subtracted, and 'g_fitc_exp', and then be capped by the information present in Table 5, which would now occupy Columns 17–22.

4.1.2.2 Save ROI Data

The 'Save ROI Data' saves only the data, detailed above in 4.1.2.1.

4.1.2.3 Save Images Only

'Save Images Only' saves three snapshots related to the current displayed setup. This enables the user to quickly share their current analysis.

1. All Numbered Cells: an exact snapshot of the current displayed setup, including any changed channel contrasts, with each individual ROIs both outlined and numbered.
2. All Outlined Cells: an exact snapshot of the current displayed setup, including any changed channel contrasts, with each individual ROI outlined.
3. Image Snapshot: an exact snapshot of the current displayed setup, including any changed channel contrasts, without each individual ROI outlined.

Examples of each of these images are shown in Figure 10:

4.1.2.4 Save ROI Mask

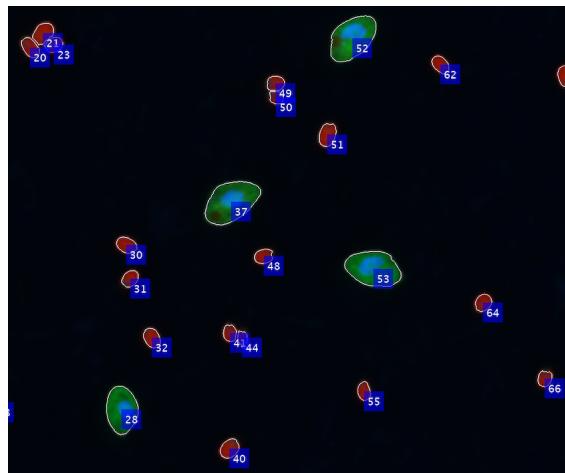
'Save ROI Mask' saves only the current existing ROI binary mask under either the image name or a user-defined name. If any ROIs have been identified as described in Section 4.2.17, the ROI IDs are saved in the created .mat file as well. Upon initially selecting this option, the user will be prompted to either enter a name or to save the ROI mask under the default image name. Once this selection has been made, the user will not be prompted again for the duration of the session. This saved mask can be loaded onto the current image in future sessions using the 'Load Mask' feature defined in Section 4.2.15. If any ROI IDs exist in the saved mask, the user will be prompted as to whether they would like to load the found ROI IDs.

4.1.2.5 Save Outlined Cells Image

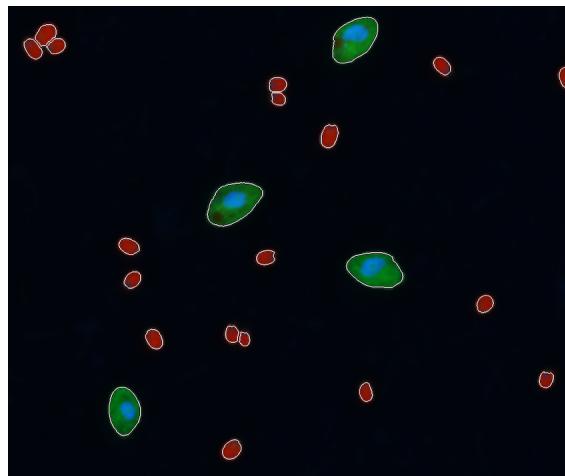
'Save Outlined Cells Image' saves only the 'Outlined Cells' image defined in Figure 10. This image is an exact snapshot of the current displayed setup, including any changed channel contrasts, with any ROI outlines. This image can be useful for sharing current analyses or using in presentations.

4.1.2.6 Save ROI IDs

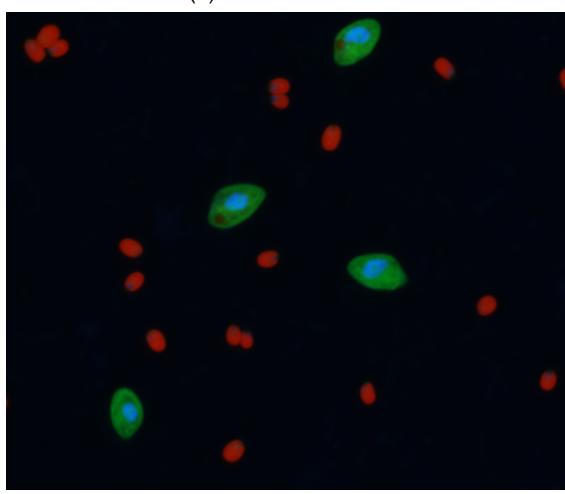
'Save ROI IDs' only becomes enabled once at least one ROI has been identified. Once it's been enabled, you can select this file menu option to save all ROI IDs as a .csv file in the selected output directory. All ROI IDs, regardless of whether they have been identified or not, are included in the export. Any and all undefined ROIs, i.e ROIs that were NOT marked as 'Other' or a specific type, are listed as 'Undefined'.



(a) Numbered Cells



(b) Outlined Cells



(c) Image Snapshot

Figure 10: Exported Images with 'Save Images'

4.1.3 Undo ROI

'Undo ROI' returns the defined ROI binary mask to its previous state as defined in the current program session. All previous ROI states are lost upon ending a session, except for the most recent binary mask. Read more about the autosaved mask and its recovery in Section 4.2.16, 'Load Autosaved Mask'.

4.1.4 Redo ROI

'Redo ROI' is only an option as long as one's most recent action was to 'Undo ROI'. This allows the user to move between all existing ROI states, either 'Undo' to move back a state or 'Redo' to move forward a state. As soon as another ROI is defined or deleted, 'Redo' is no longer an option, and all 'forward' ROI binary mask states are lost. All ROI states are lost upon ending a session, except for the most recent binary mask. Read more about the autosaved mask and its recovery in Section 4.2.16, 'Load Autosaved Mask'.

4.1.5 Export As

'Export As' allows the user to select how the ROI data will be exported. The available options are:

1. Csv
2. Text
3. Excel

Data is saved in identical rows and columns in each case as defined in Section 4.1.2.1, 'Save ROI Data & Masks'.

4.1.6 Exit

'Exit' enables the user to exit the program. It is important to note that the program will not ask the user to confirm if they would like to exit; it will simply exit immediately. The defined ROIs, however, will not be lost; each ROI creation or deletion creates an updated, autosaved ROI binary mask. This can be reloaded onto the initial image as defined in Section 4.2.16, 'Load Autosaved Mask'.

4.2 ROI Tools Menu

The ROI Tools menu, second item in the Image Analysis menu bar, holds all of the available options for defining ROIs, as well as loading pre-existing binary masks, editing the threshold channel, and changing ROI connectivity values. This menu will not become enabled until an image has been loaded.

4.2.1 Threshold Channel

'Threshold Channel' produces a side-menu list selection of channel options for thresholding ROIs. The default selection is 'All', but the user can also choose to threshold on a specific color channel. Threshold selection takes effect when using the options Auto Threshold ROI, Manual Threshold ROI,

Manual Threshold Region of ROIs, and Manual Threshold All ROIs. Thresholding values are based on the selected color channel and calculated using Otsu's method. See [Otsu's Method and Implementation](#) for specific algorithm functionality.

4.2.2 Connectivity

The connectivity affects the number of objects found in an image and the boundaries of those objects. For example, if you specify a 4-way pixel connection, the binary image below (Fig. 11) contains two objects; however, if you specify an 8-way pixel connection, the image below has one object.

0	0	0	0	0	0
0	1	1	0	0	0
0	1	1	0	0	0
0	0	0	1	1	0
0	0	0	1	1	0

Figure 11: Example Binary ROI Representation

A 4-way connection will result in more linearly defined (and generally more) ROIs, while an 8-way connection will result in generally fewer ROIs.

It is important to note that connectivity can be changed mid-image on a case-by-case basis. For instance, the user could 'Manual Threshold All ROIs' first with a 4-way connection to identify as many as possible, and then zoom in on specific regions or not-well-defined ROIs and redefine with an 8-way selected connectivity.

4.2.3 Background Subtraction

'Background Subtraction' opens a separate window tool for performing a rolling-ball background subtraction on individual channels. An example background subtraction window is shown in Figure 12.

The Background Subtraction tool operates via MATLAB's `imopen` command, a morphological image opening procedure. This takes a given shape, defined by the user, and applies it over and over again throughout the image in 'neighborhoods' within the shape. All image information within the individual shapes is analyzed on a case-by-case basis and compiled into one image mask; that mask defines the 'background' of the image, which is then subtracted from the unedited image to create a background subtracted image.

Important notes related to background subtraction:

1. Any applied background subtraction will be reflected immediately in the primary image window. However, background subtractions can be un-done, re-done, or removed at any time during an image analysis session.
2. Upon data export, both the original data (non-background subtracted) and background subtracted data will be exported.
3. When using this feature, the individual channels are shown in grayscale images rather than false color images.

- If the user swaps channels in the primary image analysis tool, such as switching the current green channel with the current red channel, this will result in a removal of any applied background subtraction from the channels involved in the swap. This will NOT immediately update the preview of an already-open Background Subtraction window; you will need to re-select a channel to update the display.

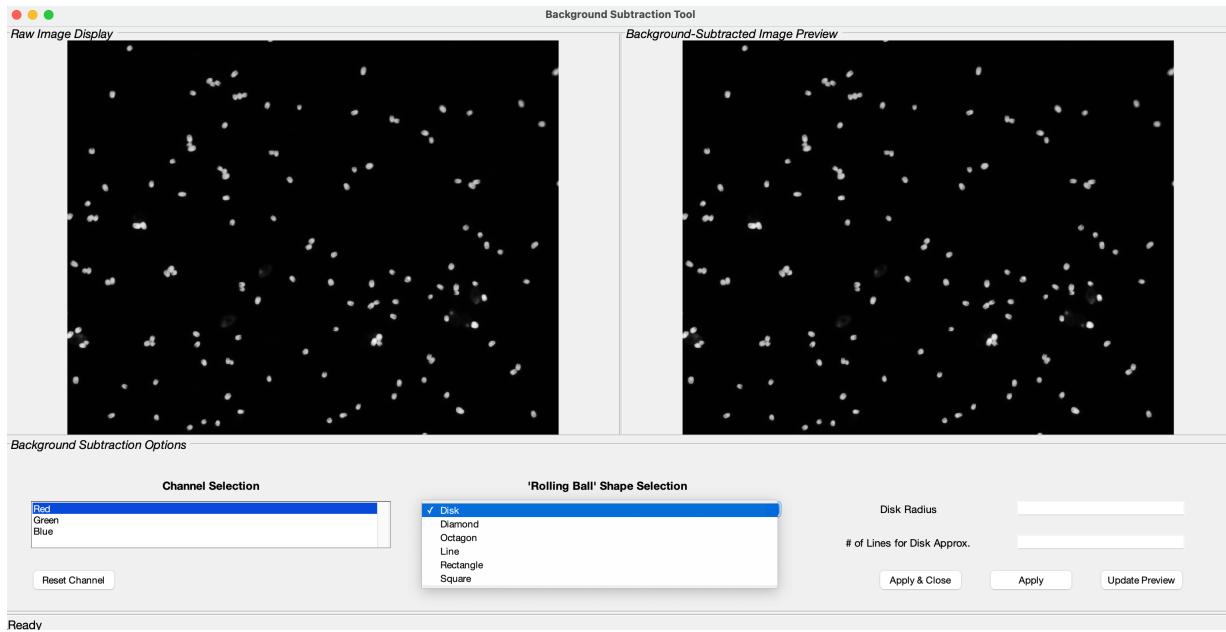


Figure 12: Background Subtraction: Example Window with Rolling Ball Dropdown

There are six available options for the rolling ball shape selection, many with similar input arguments:

- disk:** Uses a disk-shaped structuring element, with two inputs, disk radius and number of lines for the disk approximation. Disk radius is the distance from the center to the edge of the disk in pixels. Number of lines n for disk approximation refers to how many line elements MATLAB will use to approximate the disk. The allowable arguments are 0, 4, 6, and 8. Higher n creates more inclusive approximations. An n of 0 indicates no approximation, and MATLAB will include every pixel within the specified disk radius.
- diamond:** Uses a diamond-shaped structuring element with one input, diamond radius. Diamond radius is the distance from the center of the diamond to the points of the diamond in pixels.
- octagon:** Uses an octagonal structuring element with one input, describing the distance in pixels from the structuring element center to the sides of the octagon, as measured along the horizontal and vertical axes. This input must be a nonnegative multiple of 3.
- line:** Uses a line structuring element symmetric with respect to its origin. Requires two inputs; length and angle. Length is the total length of the line element in pixels, and angle, measured in degrees, is the counterclockwise rotation of the line with respect to the horizontal axis.
- rectangle:** Uses a rectangular structuring element. Requires two inputs; one with the number of rows in pixels, and another for the number of columns in pixels of the created element.

6. **square**: Uses a square structuring element, requiring only one input, the width of the square in pixels.

There are also four buttons on the Background Subtraction Tool window; "Reset Channel", "Apply & Close", "Apply", and "Update Preview".

1. **Reset Channel**: This option removes any applied background subtraction from the selected channel.
2. **Apply & Close**: Takes the selected rolling ball shape and associated inputs and performs the background subtraction procedure on the selected channel of the image currently under analysis in the primary tool. It then closes the Background Subtraction Tool window.
3. **Apply**: Takes the selected rolling ball shape and associated inputs and performs the background subtraction procedure on the selected channel of the image currently under analysis in the primary tool. Leaves the tool open to continue subtraction procedures on other channels.
4. **Update Preview**: This button applies the selected rolling ball algorithm and associated inputs to the left-hand raw image and updates the right-hand preview image to reflect the changes. This does not apply the selected background subtraction to the channel of the image currently under analysis in the primary tool.

Using the red 'X' in the top-right of the tool window to exit the tool closes the tool window without applying any background subtraction.

4.2.4 Auto Threshold ROI

'Auto Threshold ROI' (Ctrl+A) defines the largest ROI within a user-selected region based on an automatically defined threshold value. After selection, the user is prompted to define the region in which to create the ROI. After drawing a freehand region on the displayed image, the user can double-click to indicate that the region has been defined (Fig. 13). The program then internally defines all ROIs within the region that meet the automatically determined threshold value (using Otsu's Method as referenced in Section 4.2.1), and filters the ROIs based on size. Only the largest ROI is saved and outlined (Fig. 14).

4.2.5 Manual Threshold ROI

'Manual Threshold ROI' (Ctrl+W) follows a very similar process as that of 'Auto Threshold ROI' as defined in Section 4.2.4. The user is prompted to draw a freehand region in which to define ROIs, and double-clicks to finalize region selection (Fig. 15). A manual threshold interface is then created, as shown in Fig. 16. This interface allows the user to manually adjust the threshold level at which the program defines ROIs. The displayed initial value is based on the automatically defined threshold (Fig. 17). After adjustment, the program internally defines all ROIs within the region that meet the manual threshold value, and filters the ROIs based on size (Fig. 18). The largest ROI is temporarily saved and outlined, until the outlines are permanently saved using 'Confirm Outlines' (Fig. 19). Manual thresholding can often produce better ROI definition with fine control over the threshold value.

An additional, advanced option exists for defining a minimum pixel cutoff size. This sets the lower limit for pixel count for any defined ROIs. All displayed ROIs defined by manual thresholding are then

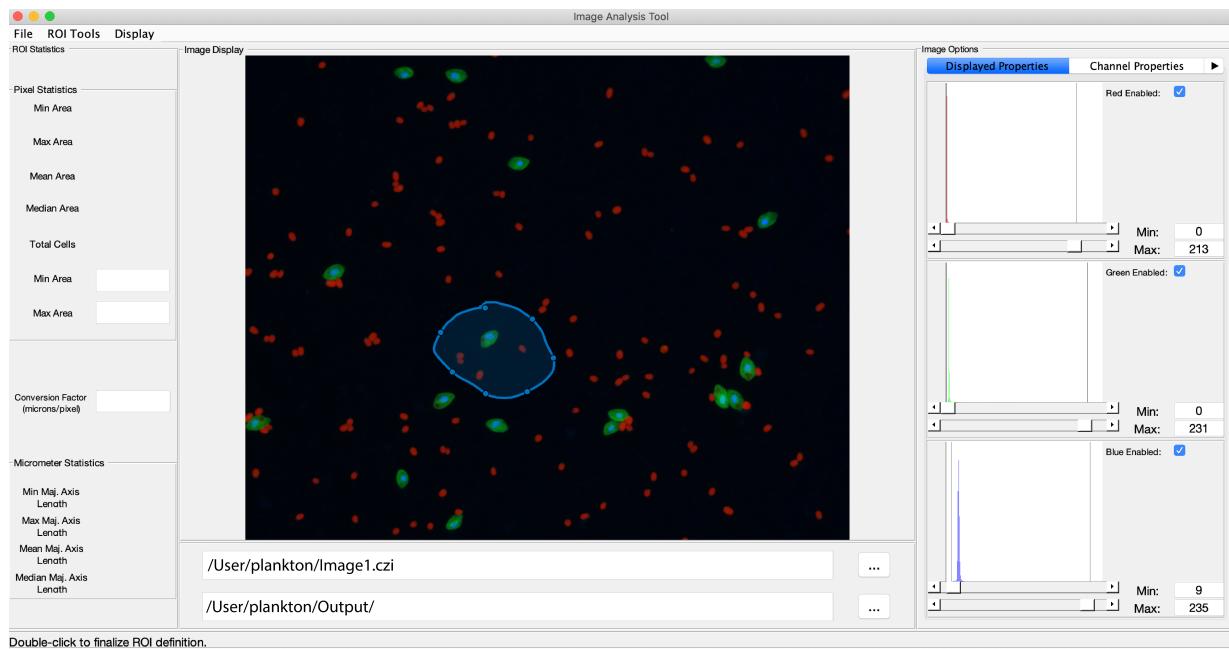


Figure 13: Auto-Threshold ROI: Defining Region

filtered by this minimum pixel count value. The specific operational function of this is further explained as input variable 'P' in MATLAB's function '[bwareaopen](#)'.

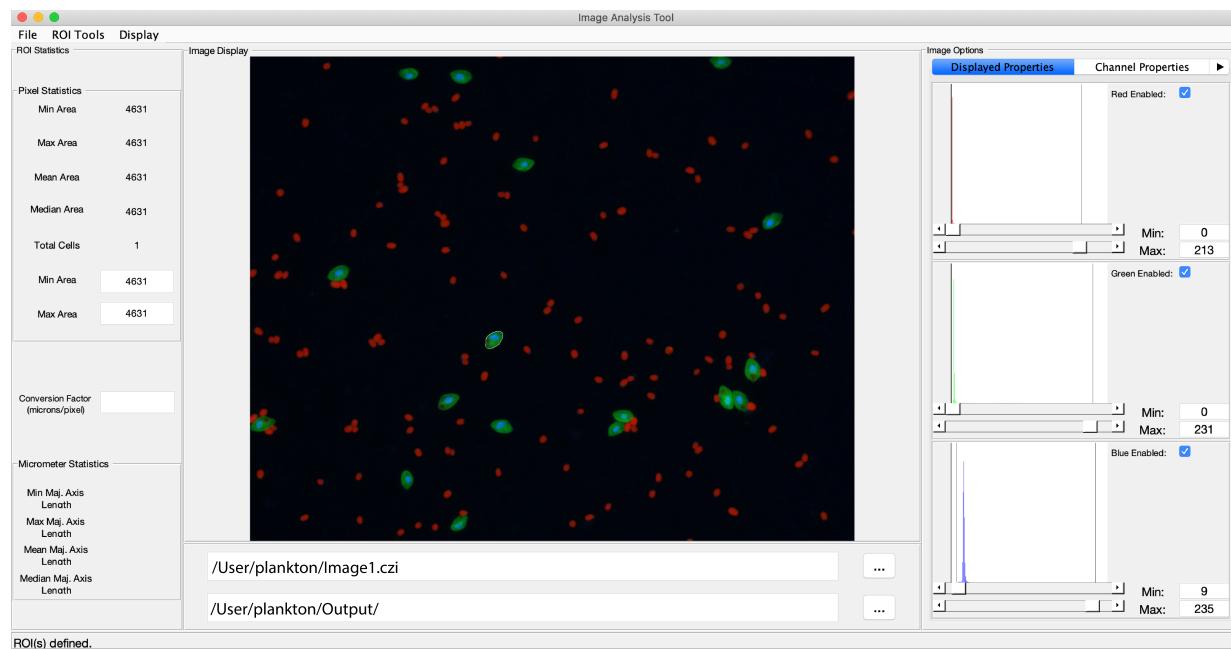


Figure 14: Auto-Threshold ROI: ROI Defined

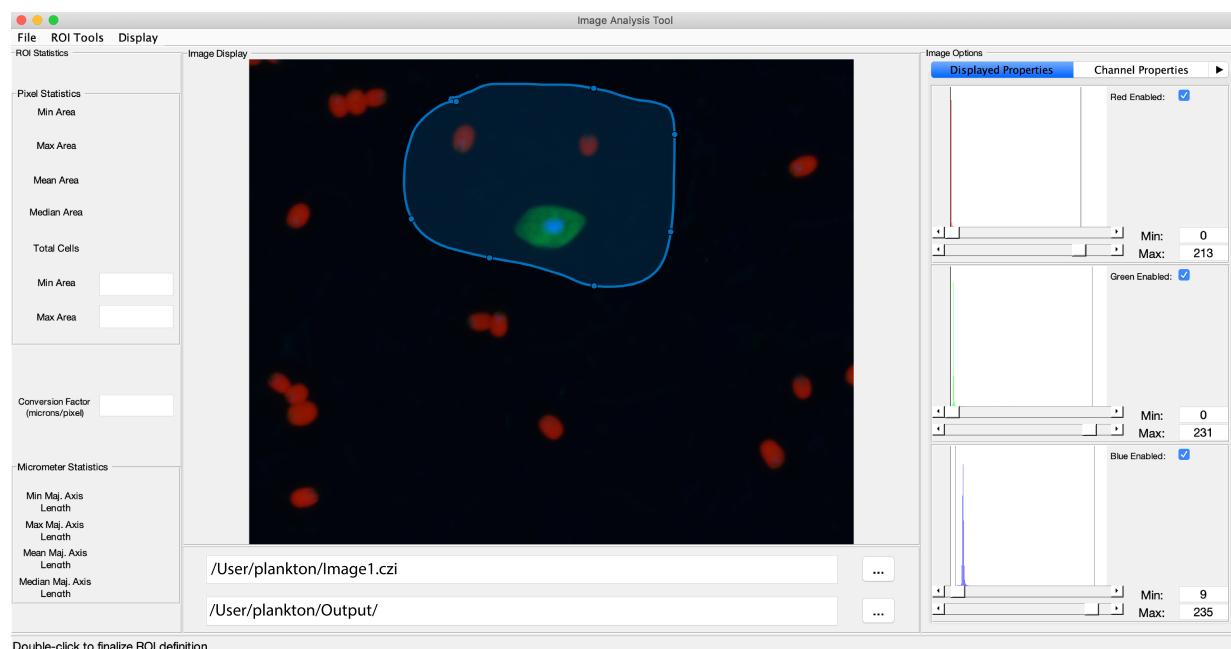


Figure 15: Manual-Threshold ROI: Defining Region

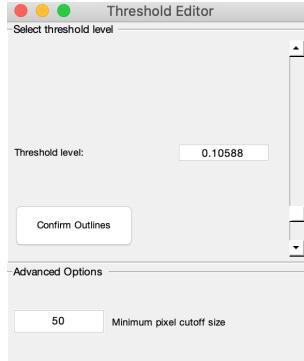


Figure 16: Manual-Threshold ROI: Manual Threshold Interface

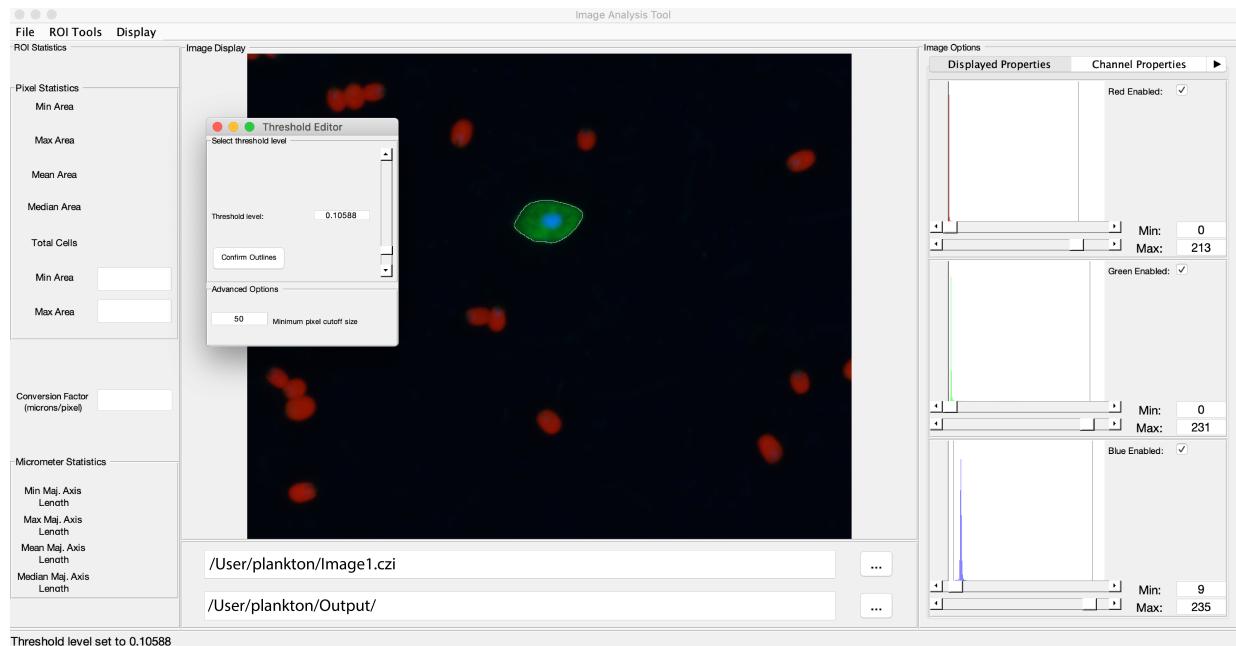


Figure 17: Manual-Threshold ROI: ROI Initial Threshold

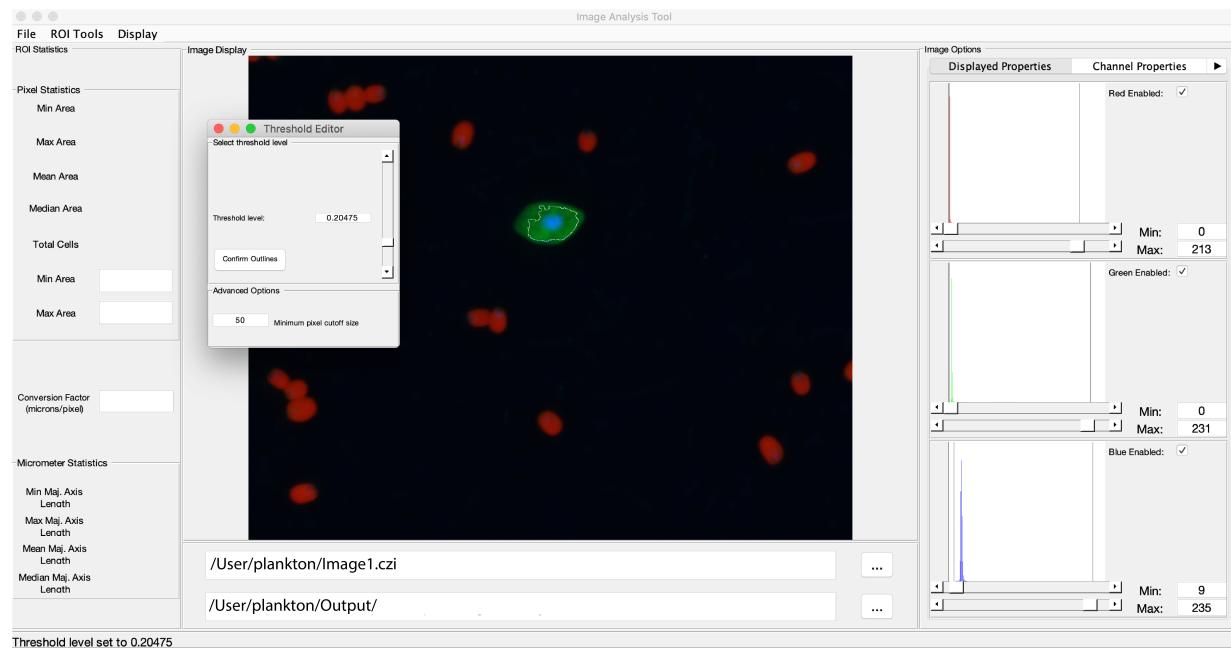


Figure 18: Manual-Threshold ROI: ROI Adjusted Threshold

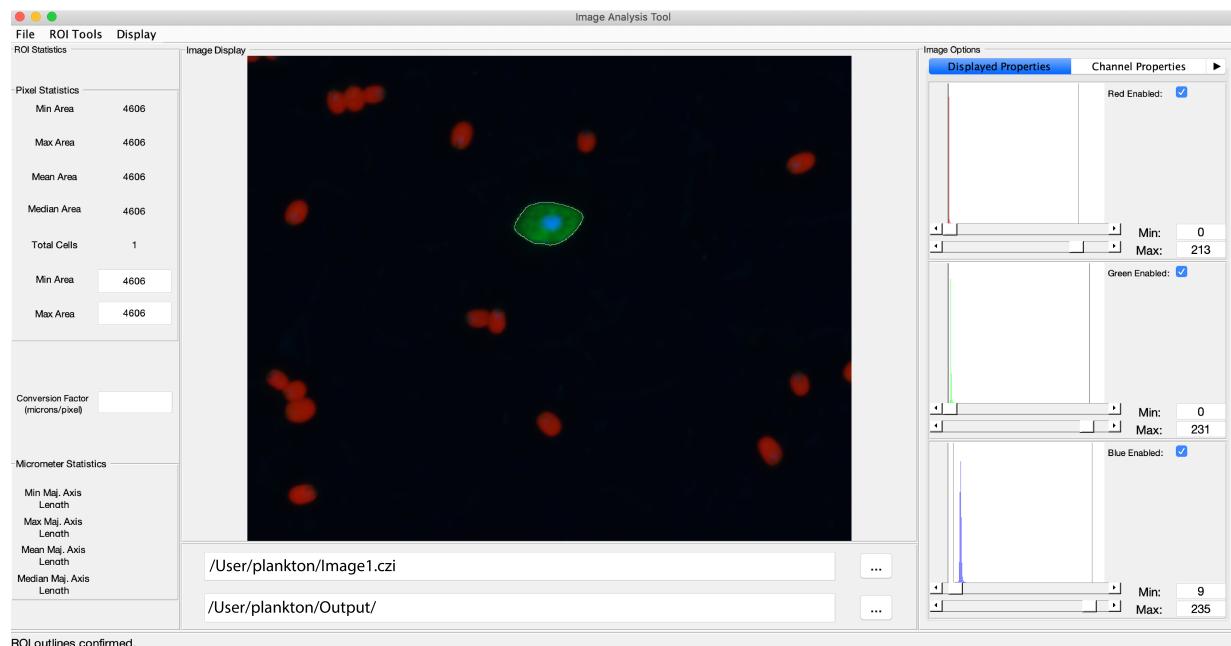


Figure 19: Manual-Threshold ROI: ROI Defined

4.2.6 Auto Threshold Region of ROIs

'Automatic Threshold a Region of ROIs' works like a combination of 'Manual Threshold a Region of ROIs' and 'Automatic Threshold a ROI', as defined in Section 4.2.7 & Section 4.2.4 respectively. The user is prompted to draw a freehand region on the image. After fully defining that freehand region, the user then double-clicks the left button to finalize the region definition. Since only a small region has been created, the Otsu's method determination of threshold level for that region becomes region-specific, much more accurate than a thresholding of the entire image. Unlike 'Automatic Threshold a ROI', this method does not filter the ROIs by size and limit the ROI selection to a single ROI, but rather includes all ROIs within the selected region in the final result. For reference, the threshold method used here is MATLAB's `graythresh`, and the freehand drawing function, as well as its many configuration options, is MATLAB's `drawfreehand`.

4.2.7 Manual Threshold Region of ROIs

'Manual Threshold Region of ROIs' (Ctrl+R) follows a very similar process as that of 'Manual Threshold ROI' as defined in Section 4.2.5. The user is prompted to draw a freehand region in which to define ROIs, and double-clicks to finalize region selection (Fig. 20). A manual threshold interface is then created, as shown in Fig. 16. This interface allows the user to manually adjust the threshold level at which the program defines ROIs. The displayed initial value is based on the automatically defined threshold (Fig. 21). After adjustment, the program internally defines all ROIs within the region that meet the manual threshold value and displays their outlines in white (Fig. 22). The ROIs are temporarily saved and outlined, until the outlines are permanently saved using 'Confirm Outlines' (Fig. 23). Unlike 'Manual Threshold ROI', this method does not filter the ROIs by size and limit the ROI selection to a single ROI, but rather includes all ROIs within the region in the final result.

An additional, advanced option exists for defining a minimum pixel cutoff size. This sets the lower limit for pixel count for any defined ROIs. All displayed ROIs defined by manual thresholding are then filtered by this minimum pixel count value. The specific operational function of this is further explained as input variable 'P' in MATLAB's function '`bwareaopen`'.

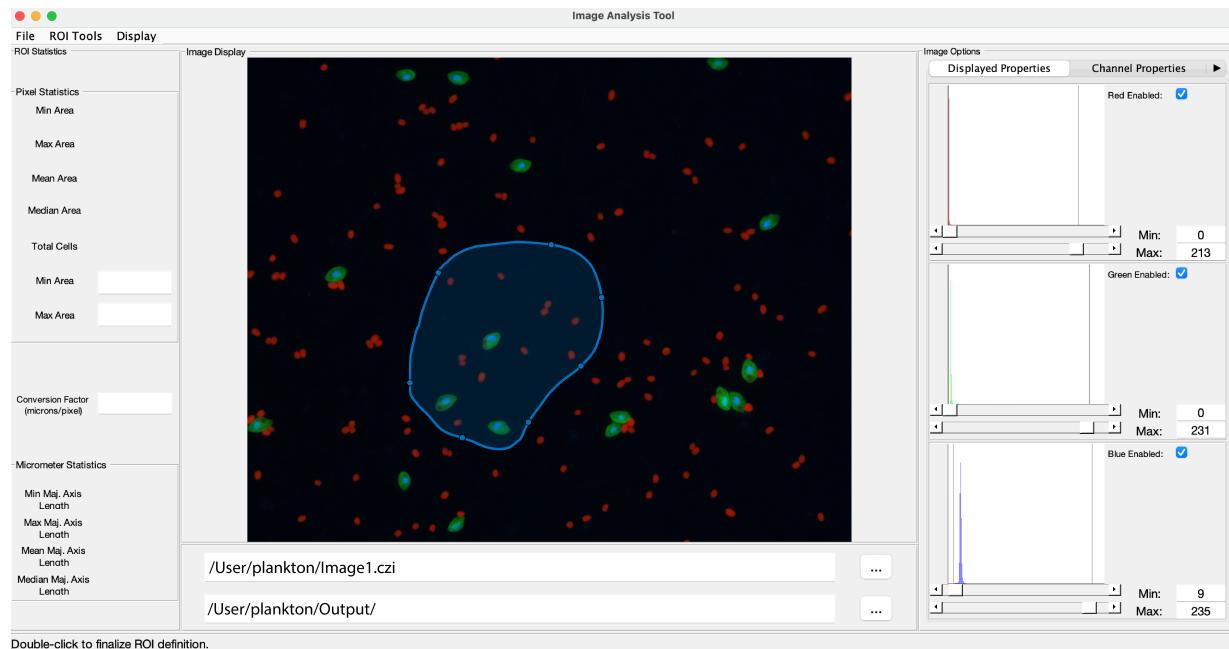


Figure 20: Manual-Threshold Region of ROIs: Defining Region

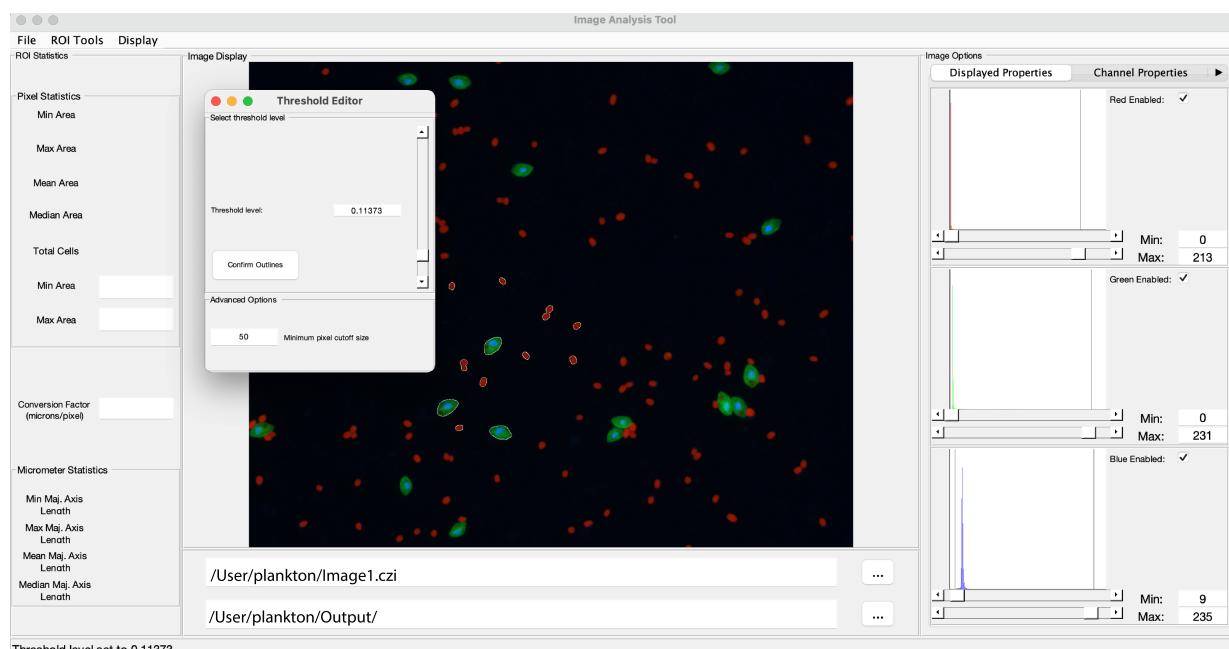


Figure 21: Manual-Threshold Region of ROIs: ROI Initial Threshold

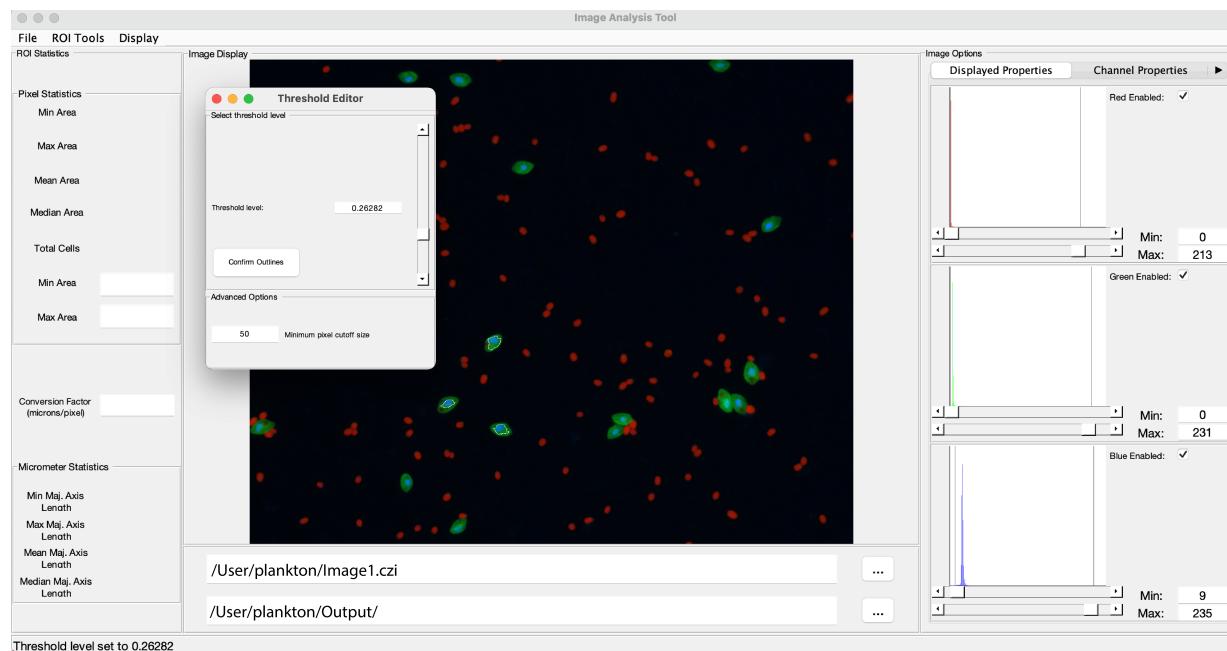


Figure 22: Manual-Threshold Region of ROIs: ROI Adjusted Threshold

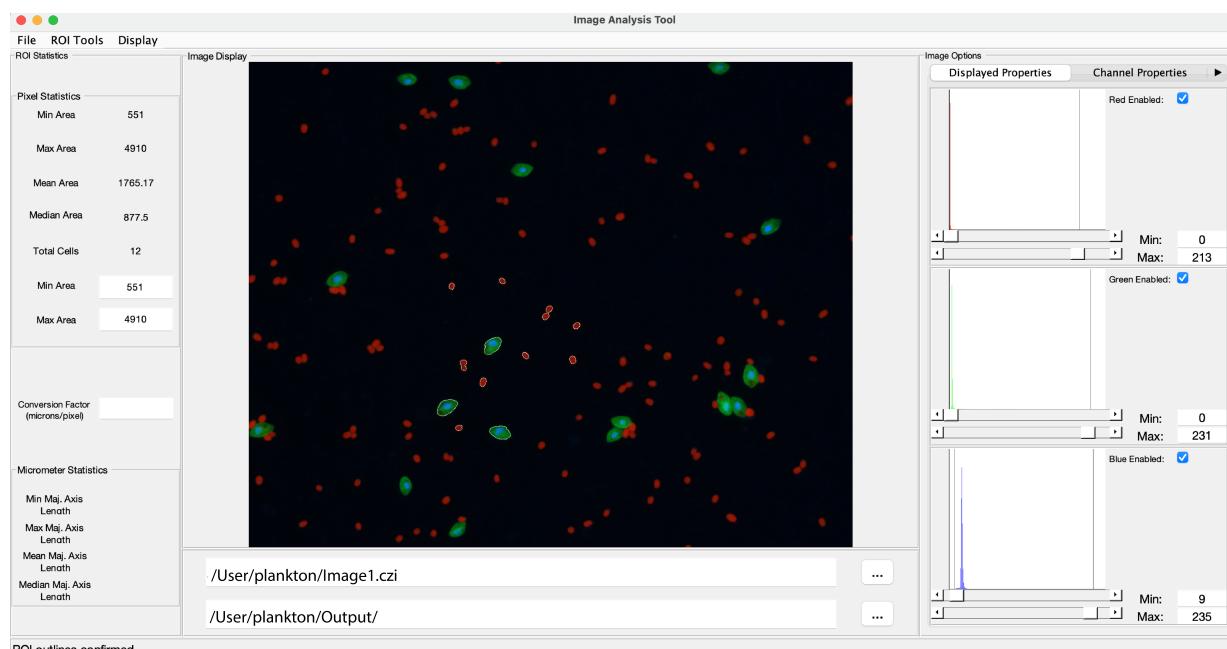


Figure 23: Manual-Threshold Region of ROIs: ROIs Defined

4.2.8 Manual Threshold All ROIs

'Manual Threshold All ROIs' follows a very similar process as that of 'Manual Threshold Region of ROIs' as defined in Section 4.2.7, except that the region over which the ROIs are defined is the entire image rather than a user-selected region. A manual threshold interface is immediately created, as shown in Fig. 16. This interface allows the user to manually adjust the threshold level at which the program defines ROIs. The displayed initial value is based on the automatically defined threshold (Fig. 24). After adjustment, the program internally defines all ROIs within the image that meet the manual threshold value and displays their outlines in white (Fig. 25). The ROIs are temporarily saved and outlined, until the outlines are permanently saved using 'Confirm Outlines' (Fig. 26).

An additional, advanced option exists for defining a minimum pixel cutoff size. This sets the lower limit for pixel count for any defined ROIs. All displayed ROIs defined by manual thresholding are then filtered by this minimum pixel count value. The specific operational function of this is further explained as input variable 'P' in MATLAB's function '[bwareaopen](#)'.

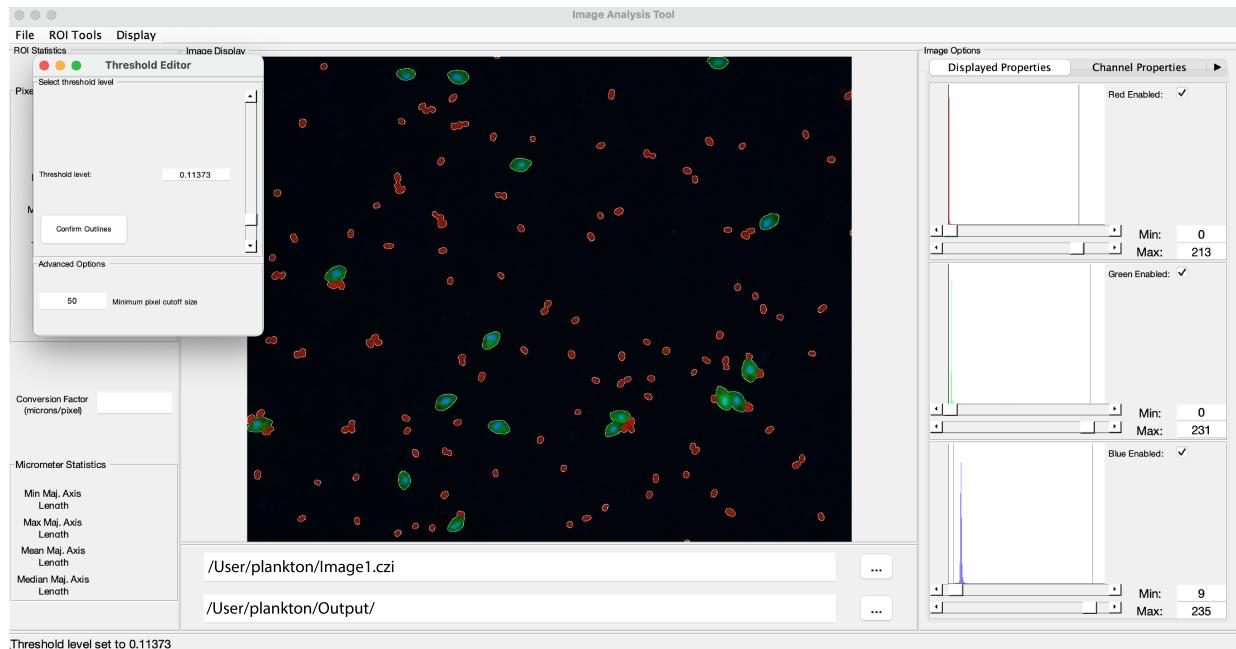


Figure 24: Manual-Threshold All ROIs: ROI Initial Threshold

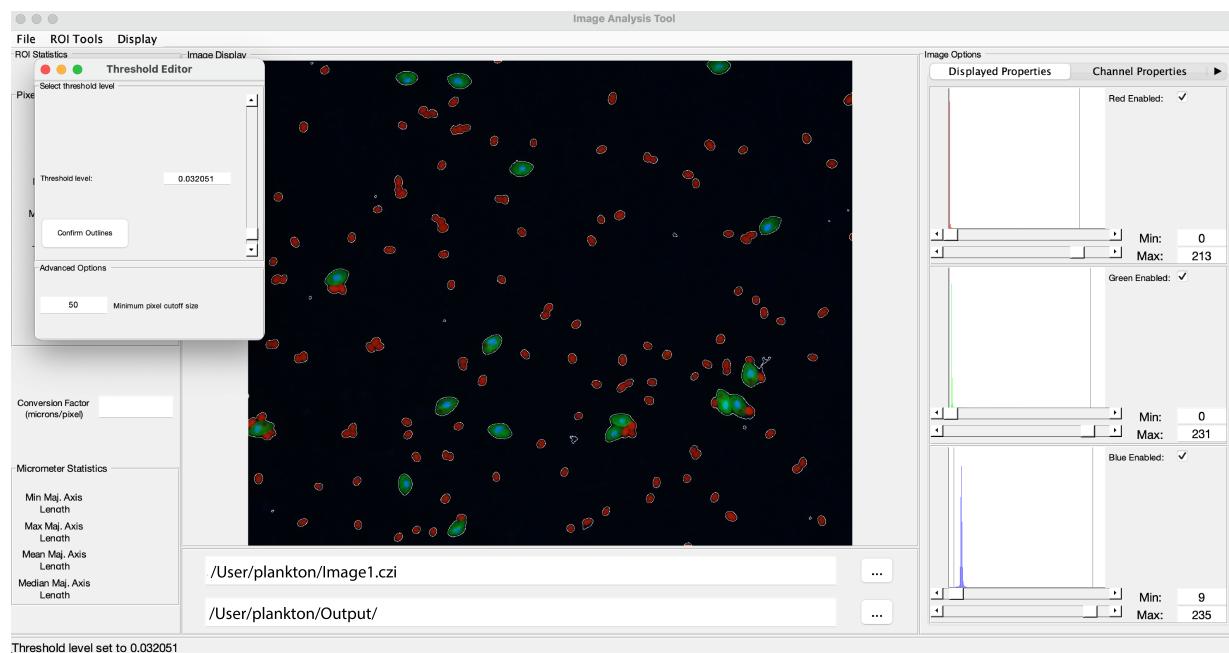


Figure 25: Manual-Threshold All ROIs: ROI Adjusted Threshold

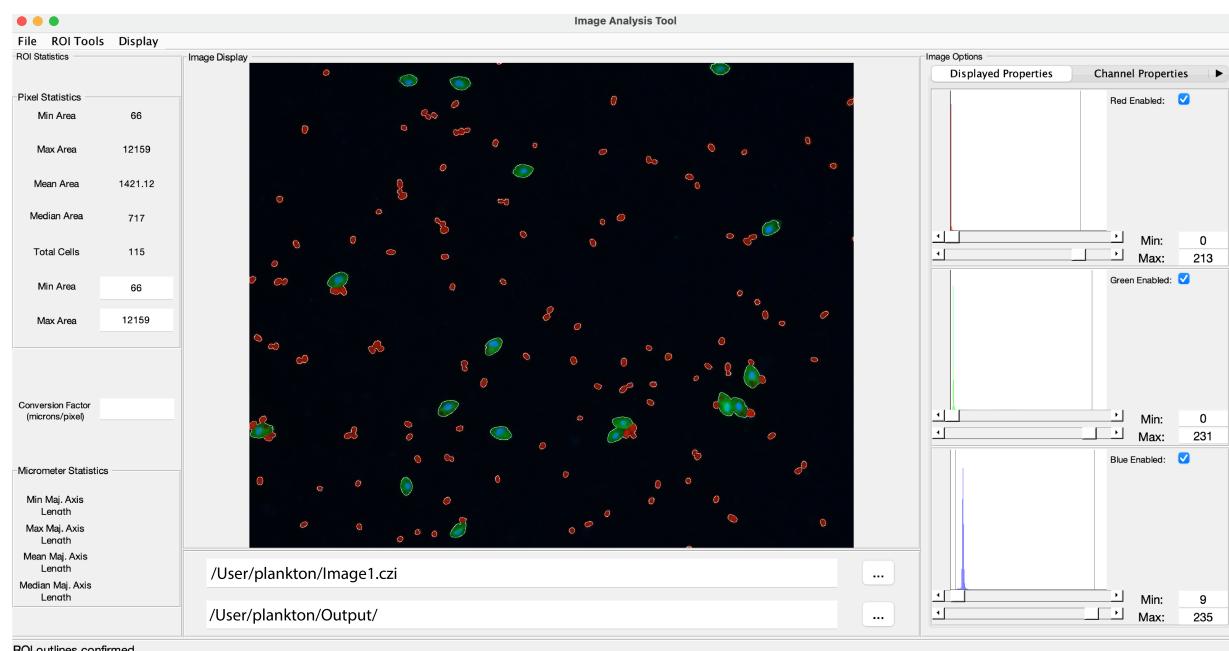


Figure 26: Manual-Threshold All ROIs: ROIs Defined

4.2.9 Draw Ellipse ROI

'Draw Ellipse ROI' (Ctrl+E) allows the user to draw a modifiable, elliptical ROI, employing MATLAB function 'drawellipse'.

After selecting the menu option, the status bar will display 'Draw ROI Ellipse' and your cursor will change into a cross hair. Click anywhere on the image to start defining your ellipse. At first only a blue dot will pop up; you can change its size and axes lengths as well as rotate it. Double-click on the ROI to finalize its definition.

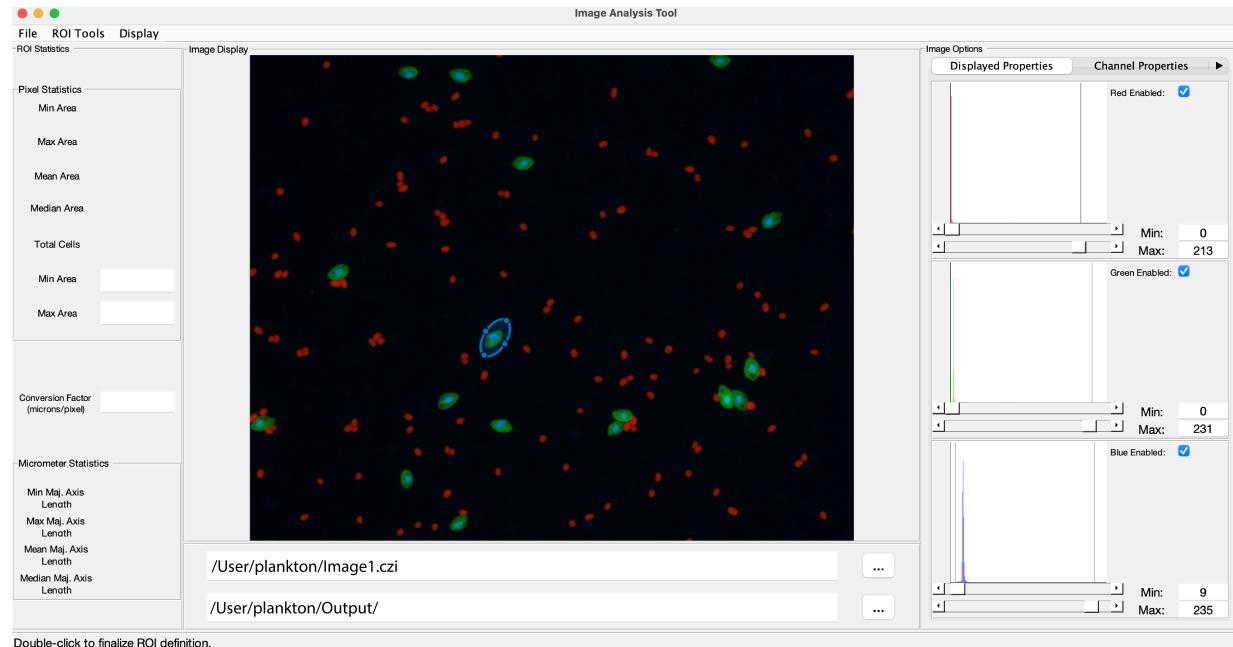


Figure 27: Defining an Ellipse

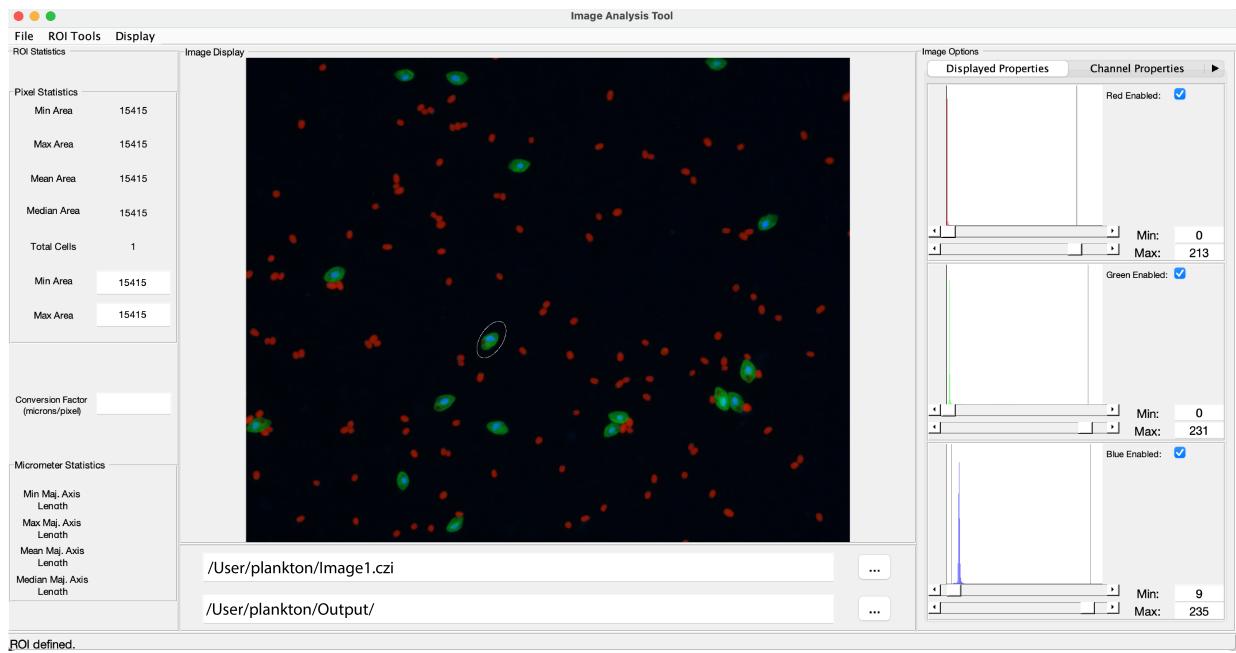


Figure 28: Ellipse Defined

4.2.10 Draw Freehand ROI

'Draw Freehand ROI' (Ctrl+F) allows the user to draw a modifiable, freehand ROI, employing MATLAB function 'drawfreehand'.

After selecting the menu option, the status bar will display 'Draw ROI Freehand' and your cursor will change into a cross hair. Click anywhere on the image to start defining your freehand region. You can draw it any way you want and as soon as you release the mouse, the last endpoint will automatically connect to the first. You can move any of the created way-points to change the freehand region, as well as left-click on a way-point to delete it. You can additionally left-click on an empty section of the border to add a modifiable way-point. Double-click on the ROI to finalize its definition.

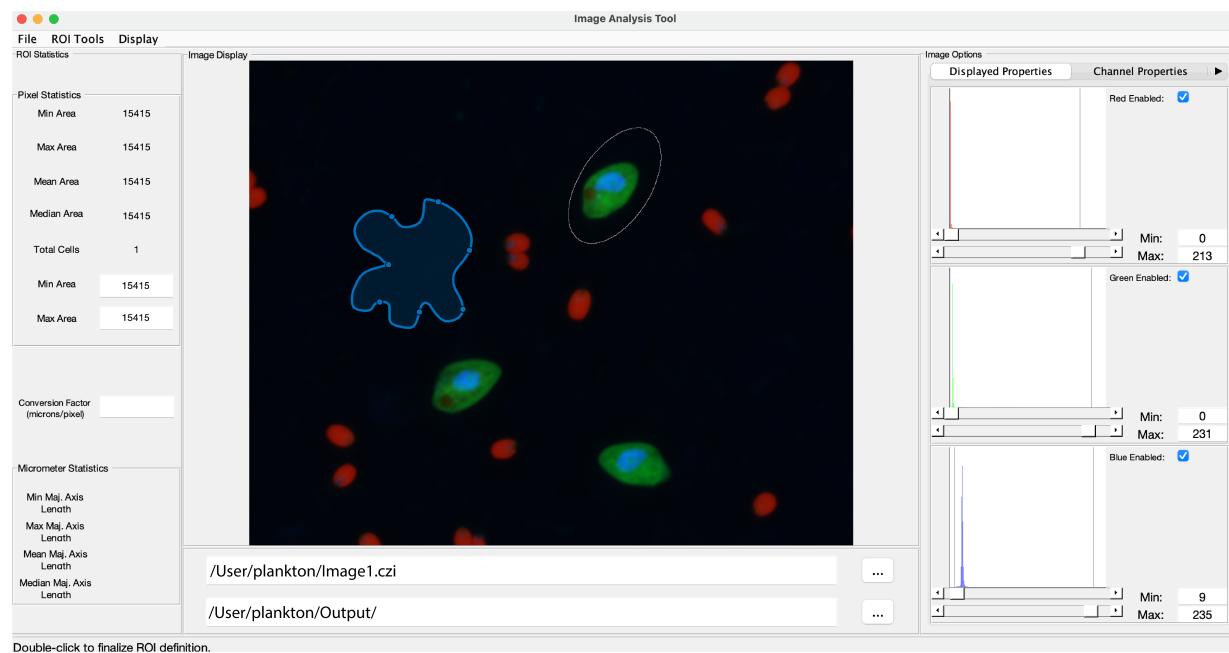


Figure 29: Defining a Freehand

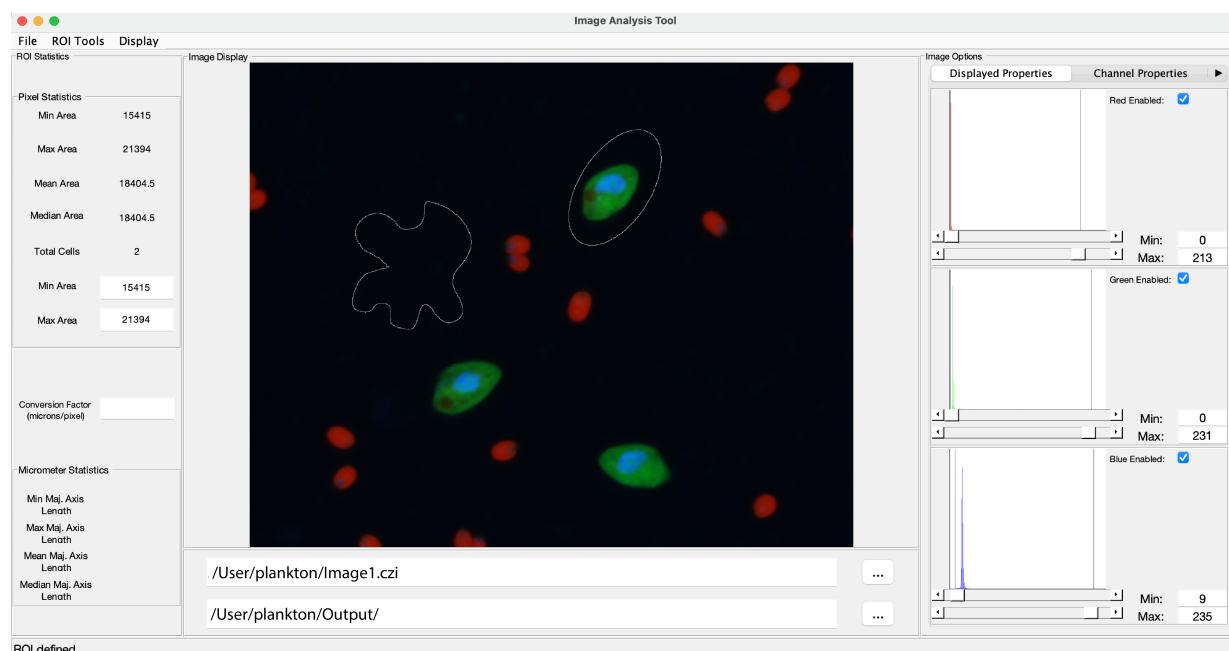


Figure 30: Freehand Defined

4.2.11 Split ROI

'Split ROI' (Ctrl+X) allows the user to draw a modifiable, freehand line, dividing any ROI it intersects. This employs MATLAB function 'drawfreehand', exactly like that of Section 4.2.10.

After selecting the menu option, the status bar will display 'Double-click to finalize split' and your cursor will change into a cross hair. Click anywhere on the image to start defining your freehand region. You can draw it any way you want and as soon as you release the mouse, the last endpoint will automatically connect to the first. You can move any of the created way-points to change the freehand region, as well as left-click on a way-point to delete it. You can additionally left-click on an empty section of the border to add a modifiable way-point. However, the split ROI function works best when you keep the freehand region as straight of a line as possible. If the cell does not split properly, Ctrl+U returns your mask to its previous state as defined in Section 4.1.3. Double-click on the split to finalize it.

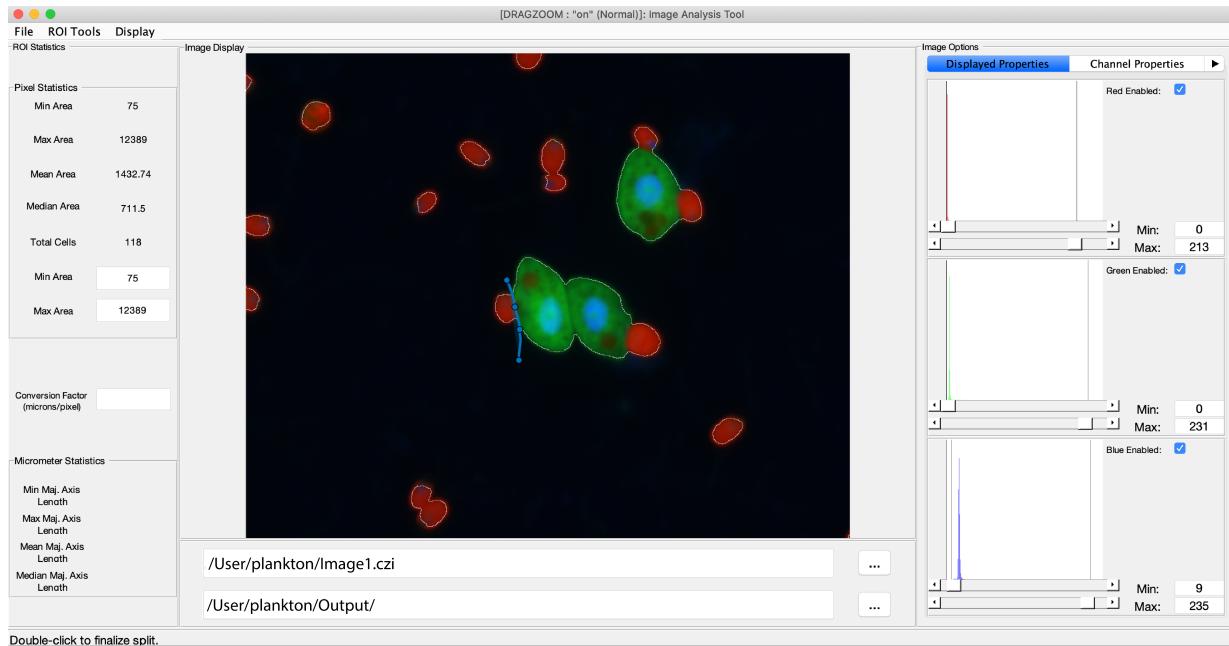


Figure 31: Splitting an ROI

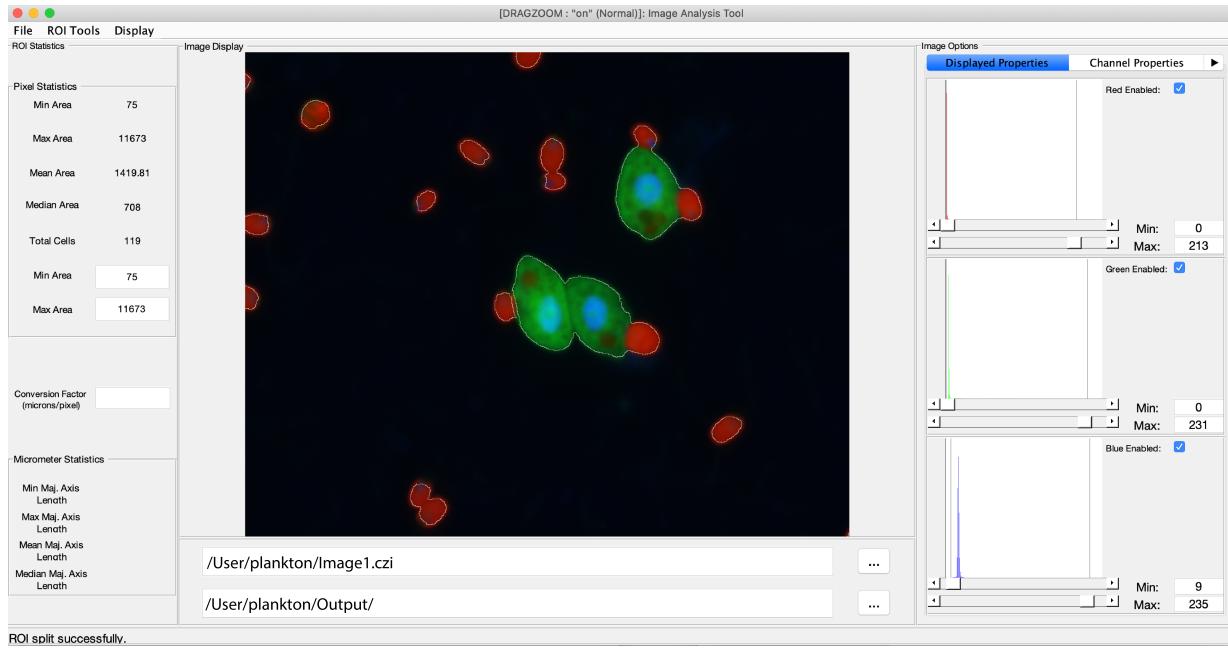


Figure 32: Split Finalized

4.2.12 Delete ROI

'Delete ROI'(Ctrl+D) allows the user to delete a single ROI by manual selection. The ROI directly below your cursor after a single click will be deleted.

After selecting the menu option, the status bar will display 'Select ROI to delete' and your cursor will change into a screen-wide crosshair. Click on any ROI to remove it. You can cancel ROI deletion by hitting the 'Escape' key or by clicking anywhere on the image that isn't an ROI. If you delete an ROI by accident, Ctrl+U returns your mask to its previous state as defined in Section 4.1.3.

4.2.13 Delete Region of ROIs

'Delete Region of ROIs' allows the user to delete all ROIs, or sections of an ROI, by defining a freehand region. Every ROI or section of an ROI within the defined freehand region will be deleted. The freehand definition employs MATLAB function 'drawfreehand', exactly like that of Section 4.2.10.

After selecting the menu option, the status bar will display 'Draw ROI Freehand' and your cursor will change into a cross hair. Click anywhere on the image to start defining your freehand region. You can draw it any way you want and as soon as you release the mouse, the last endpoint will automatically connect to the first. You can move any of the created way-points to change the freehand region, as well as left-click on a way-point to delete it. You can additionally left-click on an empty section of the border to add a modifiable way-point. Double-click to finalize the deletion. If regional deletion does not go as planned, Ctrl+U returns your mask to its previous state as defined in Section 4.1.3.

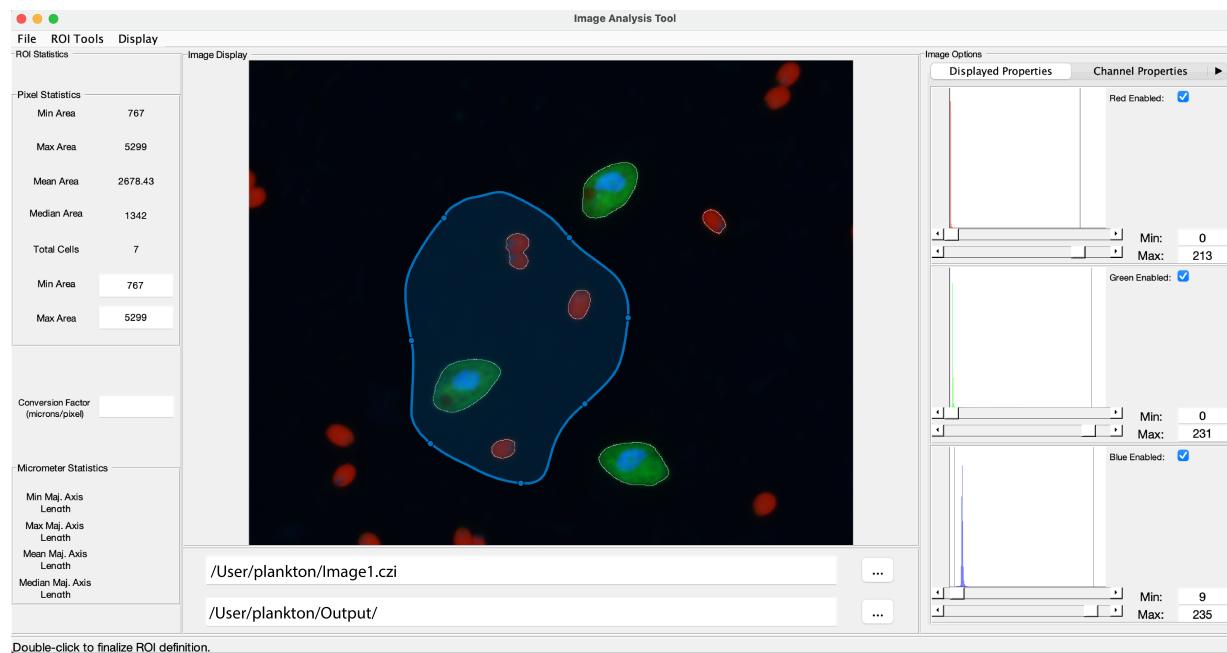


Figure 33: Define Deletion Region

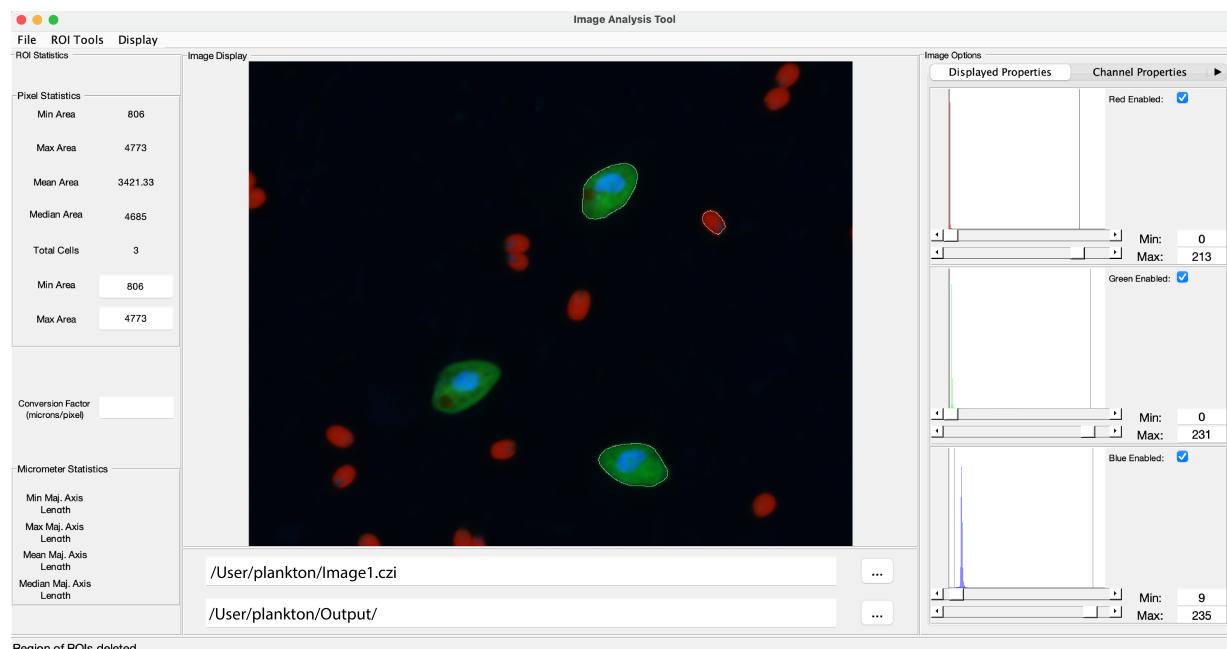


Figure 34: Delete Region of ROIs

4.2.14 Delete All ROIs

'Delete All ROIs' allows the user to delete all currently defined ROIs. Every ROI currently displayed on the image will be deleted. A selection dialog will pop up asking the user to confirm their decision. As with all of the other delete options, Ctrl+U returns your mask to its previous state as defined in Section 4.1.3.

4.2.15 Load Mask

'Load Mask' allows the user to load an existing .mat file into the program from a previous session, so long as the binary mask in the .mat file is the exact size (row # and column #) of the currently loaded image. Masks created outside of this program may not be recognized; the program expects, at the least, the .mat file to contain a structure whose first field is the same size as the binary mask.

If ROI IDs have been made, then they are included in any saved mask. As a result, the program also checks the loaded mask for an array of ROI IDs that correspond to the ROIs within the binary mask. Should any ROIs be already defined, the program will prompt the user to either keep or overwrite those existing ROIs.

4.2.16 Load Autosaved Mask

'Load Autosaved Mask' works almost identically to 'Load Mask', but does not ask for user input and instead searches the selected output directory for a programmatically-named autosaved mask file. Should the program crash for any reason, the latest ROI mask is always saved in the output directory under the image name with the appendage 'autosaved_mask'. If the new output directory differs from the one in which the user experienced the crash, the program will fail to find an autosaved mask. There are two solutions to this issue; one, choose the 'Load Mask' options instead, and navigate to the old directory. Once there, simply select the autosaved mask and load as normal. Two, change the output directory to the directory in which the autosaved mask was stored, namely the output directory in which the crash occurred, and 'Load Autosaved Mask' once again.

NOTE: If the name of the image has been changed between program sessions, the program will not be able to find the autosaved mask alone. You will need to load the mask manually, as it depends on finding a matching mask for the image name.

4.2.17 Identify ROIs

'Identify ROIs' opens a new window for setting identifiers to individual ROIs. An example of this window can be seen in Figure 35.

Description of window features:

- The image display panel appears on the right with a zoomed-in image of the first ROI to assist in identification.
- A list of potential ROI designations appears on the top-left panel. The default is 'Other', which can't be removed. Additional designations can either be entered manually, via the 'Add' button,

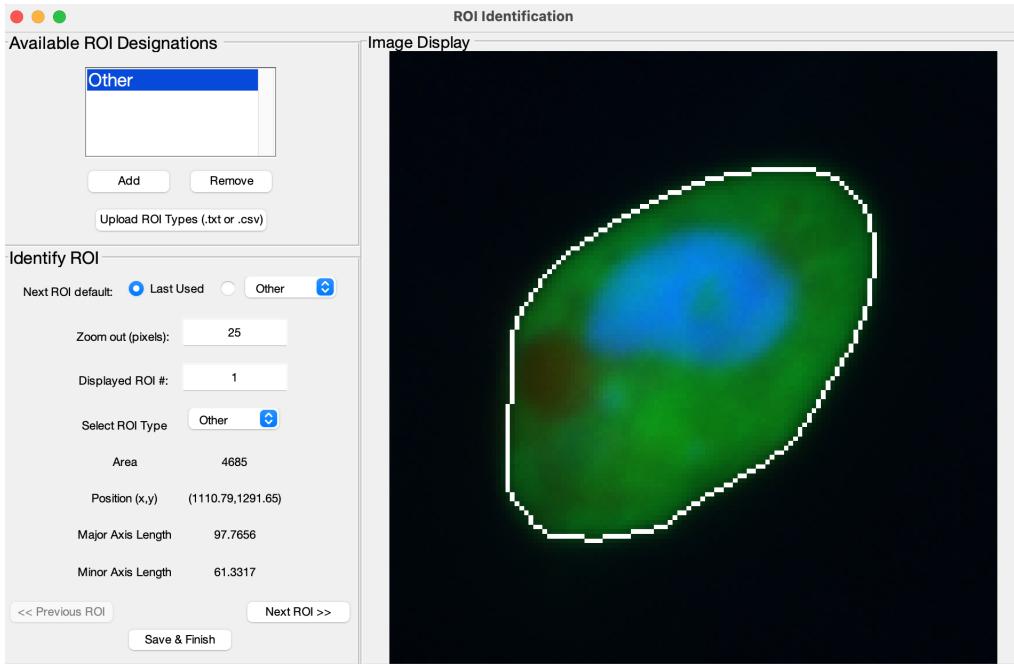


Figure 35: ROI Identification Tool

or as a bulk list text/csv file. If you choose to go the file route, there are a couple formatting considerations to ensure accurate recognition:

1. The file must be a single row or a single column.
 2. Each ROI designation must be separated by a comma.
- The 'Identify ROI' panel on the bottom left enables the user to interact with this feature.
 1. The first item in this window is the 'Next ROI Default' selection area. When moving between undefined ROIs, this selection area will decide which ROI type the ROI defaults to. The first option is the 'Last Used' which will default to the most recent ROI type the user has selected. The second option can be any of the current ROI designations, including 'Other'.
 2. The second item in this window is the 'Zoom out' text edit box, which allows the user to specify how many pixels, in each direction (up, down, left, and right) the 'Image Display' will zoom out, in case the user needs a broader perspective. The value in this box can be negative as well, allowing the user to zoom further in. Any value in this box is maintained as the user moves from ROI to ROI.
 3. The third item is the 'Displayed ROI #' indicating the number, from one to the total number of ROIs, that the current ROI represents. This box also functions as a skip-around tool, allowing the user to switch between any ROIs. A number entered below 0 defaults to 1, while any number above the total number of cells defaults to the final ROI. The more conventional way of switching between ROIs is near the bottom of the figure, indicated by the 'Previous ROI' and 'Next ROI' push buttons. As indicated, these buttons will allow the user to move to the previous or subsequent ROI.

4. The fourth item is the actual drop-down menu that allows the user to define the current ROI. When moving to any ROI that has already been defined, this selection will change to represent the identity that was selected for that ROI. When moving to any undefined ROI, this drop-down will change according to what was selected as the 'Next ROI Default'.
5. The rest of the displayed data is a series of statistics pertaining to the displayed ROI. These include area, centroid coordinates, and major and minor axis lengths, all in pixels.
6. The final button option, 'Save & Finish', saves the ROIs defined thus far and closes the tool. Simply closing the figure will also save the ROI identifications and close the tool. Any ROIs that were not visited during an ID session will get left as "Undefined" in the saved output. The ROIs that were visited will have either 'Other' if not given a user designation, or the user-given identification.

Real-time editing during ROI Identification - For ease of ROI editing, the primary ROI display on the main figure window will also track the individual ROIs as you go through them. If you spot an error, simply close the ROI ID tool and the main image window will now be zoomed into the region of the last ROI you were identifying. You can edit the ROI as normal and will not lose any ROI IDs you've made thus far. ROIs may change in numeric ID as you edit, but they will not change in designation. For instance, if you split ROI 100 into two ROIs, all ROIs after ROI 100 will be one numeric ID higher, but will maintain their designations.

If you would like to re-open the ROI identification window after editing an ROI, you have two options, 'From Start' and 'From Selection'.

1. 'From Start' will return you to the first ROI
2. 'From Selection' will create cross hairs that allow you to select a specific ROI.

4.3 Display Menu

The 'Display' menu contains all currently programmed quick-apply display editing options - zoom, default view, and reset channel contrasts. Only Zoom, due to its frequent use, has a hotkey accelerator.

4.3.1 Zoom

'Zoom' enables the user to zoom into particular regions of the image. The accelerator to activate Zoom mode is 'Ctrl + Z'. The ability to move around the image while zoomed in is based on a modified version of user Evgeny Pr's 'dragzoom' script and incorporates much of its extended abilities compared to normal MATLAB axes zoom.

The visual indicator to know you're in Zoom mode is the addition in the figure title of [DRAG-ZOOM: "on" (Normal)] (Figure 36). When Zoom mode is deactivated, this additional text will disappear.

Refer to the following lists for available mouse and hotkey options in Zoom mode.

Mouse Actions:

1. Hold and drag left button: activate panning around the image.
2. Hold and drag right button: allows the drawing of a region that the axes will zoom to.



Figure 36: Figure title when Zoom mode is on

3. Middle button (scroll wheel): increase and decrease zoom depending on direction of scroll.
4. Double-click left, right, or middle button: reset image to original view.

Hotkeys:

1. '+': increase zoom
2. '-': decrease zoom
3. 'uparrow': moves the image up, or pans downward
4. 'downarrow': moves the image down, or pans upward
5. 'leftarrow': moves the image left, or pans right
6. 'rightarrow': moves the image right, or pans left
7. 'c': changes the pointer from an 'arrow' to a 'crosshair' and includes indicators of row and column position of the crosshair on the image. Helpful when trying to find a previously defined ROI with a known centroid position.
8. 'x': if pressed and holding, mouse and hotkey functions only apply to the x-axis.
9. 'y': if pressed and holding, mouse and hotkey functions only apply to the y-axis.

Activating any ROI tool, excluding the Background Subtraction and ROI ID tools, will deactivate Zoom mode automatically. The current level of zoom will be maintained.

4.3.2 Default View

The 'Default View' option within the Display menu resets the zoom level of the image to its original level as it was on load. This option works regardless of whether or not the user is in Zoom mode.

4.3.3 Reset Channel Color Contrasts

Selecting this option resets the channel contrasts of each channel to their default values, including disabled channels, as seen in Section 4.6.1. So, for each channel, the minimum value is set to 0 and the maximum to 255, the full original range of the 8-bit displayed image.

ROI Statistics	
Pixel Statistics	
Min Area	72
Max Area	12251
Mean Area	1456.34
Median Area	747
Total Cells	115
Min Area	72
Max Area	12251
Conversion Factor (microns/pixel)	
0.21	
Micrometer Statistics	
Min Maj. Axis Length	3.8518
Max Maj. Axis Length	40.2362
Mean Maj. Axis Length	10
Median Maj. Axis Length	7.7205

Figure 37: ROI Statistics Panel

4.4 ROI Statistics Panel

The ROI statistics panel displays general statistics for all current ROIs. It has two main subsections, the Pixel Statistics and Micrometer Statistics panels (Figure 37).

The data within the Pixel Statistics panel are all in units of pixels, and include ROI area and cell information – min, max, mean, and median area, as well as the total number of independent ROIs/cells in the image. Area in this case is not an approximation; rather, every pixel within the ROI structural boundary is counted. As the boundaries themselves consist of a string of single pixels, all area values will be whole integers.

The Pixel Statistics panel also serves as a way to filter the ROIs based on size. Two text edit boxes are appended to minimum and maximum area values; by editing these values, one can limit the range of ROIs based on size.

Immediately below the Pixel Statistics panel is a text edit box to add a microscope conversion factor. With no value in this box, the Micrometer Statistics panel will remain empty. As soon as an entry is made, the Micrometer Statistics panel will populate with data.

The Micrometer Statistics panel includes converted statistics with units of microns. Rather than displaying ROI statistics of area, it displays statistics of major axis length, for a clearer perspective on general ROI shape. In this case, the micrometer statistics are an approximation, as each ROI is fitted to an ellipse and the major/minor axis length calculated from there.

4.5 Image and Output Directory Filepaths

The 'Image and Output Directory Filepaths' panel is located at the bottom of the program and displays the filepath of the displayed image as well as the output directory.

The image filepath, or the text edit box on the top of the panel with default string 'File Select...', displays the filepath of the currently loaded image. This edit box can be directly edited to load a new image; if the image is valid, the program will act as though the user has selected a new image, and follow the process of reloading the analysis. If not, the filepath will revert to the currently displayed image. One can also employ the '...' pushbutton directly to the right of the filepath text edit box to load a new image; this will act identically to that of using the File menu option 'Load Image'.

The output directory, or the text edit box on the bottom of the panel with default string 'Select Output Directory...', displays the directory path of the currently loaded image. This edit box can be directly edited to a new path; if the path is valid, the output directory will change. If the path is not valid, the displayed string itself *will not change* to a valid directory; however, a status update will display informing the user that the displayed directory is not valid, and that the most recent valid output directory will be used for data export instead. Similar to the image selection option, the '...' pushbutton directly to the right of the output directory text edit box will open a folder selection dialog allowing the user to navigate to a new output directory.

All saved data and autosaved data will be placed in the output directory selected.

4.6 Image Options Panel

The 'Image Options' panels contains three tabs holding various options for manipulating the displayed image and its properties. The three tabs can be considered in the following way:

- The 'Displayed Properties' tab affects the displayed image only, but none of the inherent properties or back-end intensities will be included in the exported data, with one important exception; if a channel is disabled, the user has the option to exclude that channel's data in the export.
- The 'Channel Properties' tab affects the inherent properties of the image and includes the ability to swap channels to different colors, as well as change the channel's name and set an exposure time.
- The 'Image Properties' tab affects nothing and serves only to display basic information of the image, such as bit size and dimensions.

4.6.1 Displayed Properties Tab

The 'Displayed Properties' tab contains options for controlling the displayed image during real-time analysis only and changes made do not influence the raw image data. One can disable channels for viewing purposes here, as well as adjust the minimum and maximum 8-bit intensity for better contrast. It is important to note that all images, regardless of their original intensity, are displayed in 8-bit. As a result, the range of intensity values for each channel will always be from 0 to 255. Exported data is in the same units as the original image; if the CZI image is 14 bits, for instance, the exported data would be in 14 bits, while the displayed image would be in 8. The plot for each channel, and the sliders below, are for better visualization as to where the highest intensities in the image lie. By adjusting the text edit boxes directly, or by adjusting the sliders, one can adjust the minimum and maximum intensity values

for each color channel individually. The plot is an image histogram plot, with an x-axis of intensity in 8-bits (0-255) and a y-axis of number of each intensity pixel within that channel.

4.6.2 Channel Properties Tab

The 'Channel Properties' tab contains options for controlling color channel assignments, channel names, and channel exposure times (Figure 5). If the image contains this information, it will automatically assign values for these options. If you want to assign channel names and channel exposure times (or change the values from what automatically loaded), simply enter the new name of the channel in the labeled text edit box, or enter a number in seconds for the text edit box under 'Exposure Time'.

NOTE: Editing channel name and channel exposure time here does not change the metadata of the image itself; it affects only the exported data. Additionally, the program currently does not use the exposure time for any analysis or intensity adjustment; all the exposure time does is change the exposure time data column for that channel in the exported data for any downstream analysis.

The primary function of the 'Selected Channel' dropdown is to allow the user to cycle through more than three image channels, as is the case with multi-image grayscale tiffs or some CZIs. For instance, in reference to Figure 39, four channels are visible in the dropdown menu. Given operating system limits, and for clarity, we are limited to three visual color channels, Red, Green, and Blue. In this case, DAPI was assigned to Blue, FITC to Green, and Cy3 to Red. However, this CZI has a fourth channel, Cy5. To view it, one would simply select the dropdown for any of the channels and select 'Cy5', and the Cy5 channel would be switched with the original channel, such as DAPI for Cy5 in Red.

In order to swap an already slotted channel with another color, first you need to change that channel to 'None' or to an unselected channel. Then, change the color channel you want the channel to be in to that channel. For example, if 'DAPI' is set to the Blue Channel and 'FITC' is to the Green Channel. To switch 'DAPI' to Green Channel, you must first switch the Blue Channel, 'DAPI' to either 'None' or 'Cy5', both currently unselected channels. Then, you can switch the Green Channel, currently 'FITC', to 'DAPI'.

Any color channel set to 'None' is not exported. All other selected channels are exported as the color they are assigned to, such as 'DAPI' to Blue and 'FITC' to Green. Additional channels, when present, are recognized on export. In the case of Figures 38 and 39, four channels are present. During data saving, the additional channel (Cy5) would be detected, and the user would be asked whether or not to include that channel in the exported data. If so, the program will then ask which color channel to assign the additional channel to. For example, if you export Cy5 as the Red channel, it will appear in the output data as r_cy5_max, r_cy5_min, etc, after the first three channels.

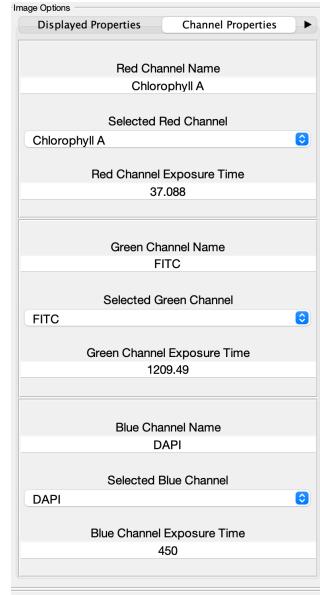


Figure 38: Channel Properties Tab

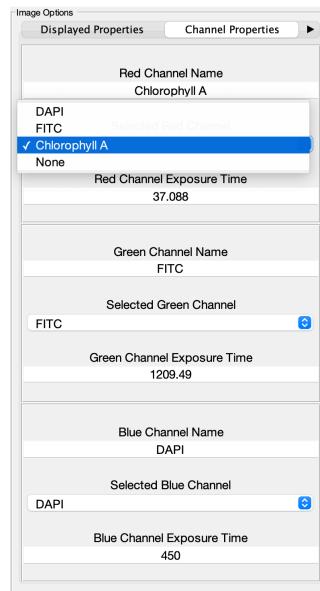


Figure 39: Example Channel Swap Dropdown

4.6.3 Image Properties Tab

The 'Image Properties' tab, as shown in Figure 40, displays basic image information for reference. No values here can be changed. Currently, this tab includes the name of the image, the type of image, the location of the image, the bit depth of the image, image dimensions, and file size.

The image location display, due to MATLAB text wrapping defaults, is one continuous line with all whitespace removed. This means that a filepath such as 'D:\Bio Matlab\image_analysis' will appear as 'D:\BioMatlab\image_analysis'. However, if one hovers over the filepath, a tool tip will appear with the full filepath *including* any whitespace characters.

Bit depth refers to the units of the image. Many CZIs have intensity value ranges of 14 or 16 bits, while most .tifs (RGBs and grayscale images) are 8-bit images. All displayed images are in or converted to 8 bits for ease of viewing and ROI identification, but all exported data is in the original intensity units of the image. MATLAB does not distinguish between an image of 14 or 16 bits, and assumes both are 'uint16' images. However, since intensity values are taken directly from the original image, this grouping does not affect exported data.

Image dimensions are displayed as 'Height x Width' in pixels, which can alternatively be thought of as 'Row x Column'.

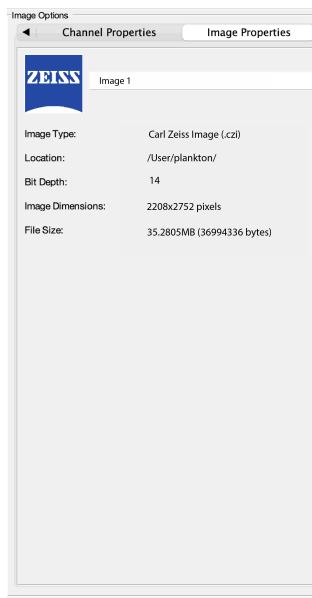


Figure 40: Image Properties Tab

5 Licensing

This program is covered under the GNU General Public License, Version 3, as reproduced below.

GNU GENERAL PUBLIC LICENSE
Version 3, 29 June 2007

Copyright (C) 2007 Free Software Foundation, Inc. <<https://fsf.org/>>
Everyone is permitted to copy and distribute verbatim copies
of this license document, but changing it is not allowed.

Preamble

The GNU General Public License is a free, copyleft license for
software and other kinds of works.

The licenses for most software and other practical works are designed
to take away your freedom to share and change the works. By contrast,
the GNU General Public License is intended to guarantee your freedom to
share and change all versions of a program--to make sure it remains free
software for all its users. We, the Free Software Foundation, use the
GNU General Public License for most of our software; it applies also to
any other work released this way by its authors. You can apply it to
your programs, too.

When we speak of free software, we are referring to freedom, not
price. Our General Public Licenses are designed to make sure that you
have the freedom to distribute copies of free software (and charge for
them if you wish), that you receive source code or can get it if you
want it, that you can change the software or use pieces of it in new
free programs, and that you know you can do these things.

To protect your rights, we need to prevent others from denying you
these rights or asking you to surrender the rights. Therefore, you have
certain responsibilities if you distribute copies of the software, or if
you modify it: responsibilities to respect the freedom of others.

For example, if you distribute copies of such a program, whether
gratis or for a fee, you must pass on to the recipients the same
freedoms that you received. You must make sure that they, too, receive
or can get the source code. And you must show them these terms so they
know their rights.

Developers that use the GNU GPL protect your rights with two steps:
(1) assert copyright on the software, and (2) offer you this License
giving you legal permission to copy, distribute and/or modify it.

For the developers' and authors' protection, the GPL clearly explains

that there is no warranty for this free software. For both users' and authors' sake, the GPL requires that modified versions be marked as changed, so that their problems will not be attributed erroneously to authors of previous versions.

Some devices are designed to deny users access to install or run modified versions of the software inside them, although the manufacturer can do so. This is fundamentally incompatible with the aim of protecting users' freedom to change the software. The systematic pattern of such abuse occurs in the area of products for individuals to use, which is precisely where it is most unacceptable. Therefore, we have designed this version of the GPL to prohibit the practice for those products. If such problems arise substantially in other domains, we stand ready to extend this provision to those domains in future versions of the GPL, as needed to protect the freedom of users.

Finally, every program is threatened constantly by software patents. States should not allow patents to restrict development and use of software on general-purpose computers, but in those that do, we wish to avoid the special danger that patents applied to a free program could make it effectively proprietary. To prevent this, the GPL assures that patents cannot be used to render the program non-free.

The precise terms and conditions for copying, distribution and modification follow.

TERMS AND CONDITIONS

0. Definitions.

"This License" refers to version 3 of the GNU General Public License.

"Copyright" also means copyright-like laws that apply to other kinds of works, such as semiconductor masks.

"The Program" refers to any copyrightable work licensed under this License. Each licensee is addressed as "you". "Licensees" and "recipients" may be individuals or organizations.

To "modify" a work means to copy from or adapt all or part of the work in a fashion requiring copyright permission, other than the making of an exact copy. The resulting work is called a "modified version" of the earlier work or a work "based on" the earlier work.

A "covered work" means either the unmodified Program or a work based on the Program.

To "propagate" a work means to do anything with it that, without

permission, would make you directly or secondarily liable for infringement under applicable copyright law, except executing it on a computer or modifying a private copy. Propagation includes copying, distribution (with or without modification), making available to the public, and in some countries other activities as well.

To "convey" a work means any kind of propagation that enables other parties to make or receive copies. Mere interaction with a user through a computer network, with no transfer of a copy, is not conveying.

An interactive user interface displays "Appropriate Legal Notices" to the extent that it includes a convenient and prominently visible feature that (1) displays an appropriate copyright notice, and (2) tells the user that there is no warranty for the work (except to the extent that warranties are provided), that licensees may convey the work under this License, and how to view a copy of this License. If the interface presents a list of user commands or options, such as a menu, a prominent item in the list meets this criterion.

1. Source Code.

The "source code" for a work means the preferred form of the work for making modifications to it. "Object code" means any non-source form of a work.

A "Standard Interface" means an interface that either is an official standard defined by a recognized standards body, or, in the case of interfaces specified for a particular programming language, one that is widely used among developers working in that language.

The "System Libraries" of an executable work include anything, other than the work as a whole, that (a) is included in the normal form of packaging a Major Component, but which is not part of that Major Component, and (b) serves only to enable use of the work with that Major Component, or to implement a Standard Interface for which an implementation is available to the public in source code form. A "Major Component", in this context, means a major essential component (kernel, window system, and so on) of the specific operating system (if any) on which the executable work runs, or a compiler used to produce the work, or an object code interpreter used to run it.

The "Corresponding Source" for a work in object code form means all the source code needed to generate, install, and (for an executable work) run the object code and to modify the work, including scripts to control those activities. However, it does not include the work's System Libraries, or general-purpose tools or generally available free programs which are used unmodified in performing those activities but which are not part of the work. For example, Corresponding Source

includes interface definition files associated with source files for the work, and the source code for shared libraries and dynamically linked subprograms that the work is specifically designed to require, such as by intimate data communication or control flow between those subprograms and other parts of the work.

The Corresponding Source need not include anything that users can regenerate automatically from other parts of the Corresponding Source.

The Corresponding Source for a work in source code form is that same work.

2. Basic Permissions.

All rights granted under this License are granted for the term of copyright on the Program, and are irrevocable provided the stated conditions are met. This License explicitly affirms your unlimited permission to run the unmodified Program. The output from running a covered work is covered by this License only if the output, given its content, constitutes a covered work. This License acknowledges your rights of fair use or other equivalent, as provided by copyright law.

You may make, run and propagate covered works that you do not convey, without conditions so long as your license otherwise remains in force. You may convey covered works to others for the sole purpose of having them make modifications exclusively for you, or provide you with facilities for running those works, provided that you comply with the terms of this License in conveying all material for which you do not control copyright. Those thus making or running the covered works for you must do so exclusively on your behalf, under your direction and control, on terms that prohibit them from making any copies of your copyrighted material outside their relationship with you.

Conveying under any other circumstances is permitted solely under the conditions stated below. Sublicensing is not allowed; section 10 makes it unnecessary.

3. Protecting Users' Legal Rights From Anti-Circumvention Law.

No covered work shall be deemed part of an effective technological measure under any applicable law fulfilling obligations under article 11 of the WIPO copyright treaty adopted on 20 December 1996, or similar laws prohibiting or restricting circumvention of such measures.

When you convey a covered work, you waive any legal power to forbid circumvention of technological measures to the extent such circumvention

is effected by exercising rights under this License with respect to the covered work, and you disclaim any intention to limit operation or modification of the work as a means of enforcing, against the work's users, your or third parties' legal rights to forbid circumvention of technological measures.

4. Conveying Verbatim Copies.

You may convey verbatim copies of the Program's source code as you receive it, in any medium, provided that you conspicuously and appropriately publish on each copy an appropriate copyright notice; keep intact all notices stating that this License and any non-permissive terms added in accord with section 7 apply to the code; keep intact all notices of the absence of any warranty; and give all recipients a copy of this License along with the Program.

You may charge any price or no price for each copy that you convey, and you may offer support or warranty protection for a fee.

5. Conveying Modified Source Versions.

You may convey a work based on the Program, or the modifications to produce it from the Program, in the form of source code under the terms of section 4, provided that you also meet all of these conditions:

- a) The work must carry prominent notices stating that you modified it, and giving a relevant date.
- b) The work must carry prominent notices stating that it is released under this License and any conditions added under section 7. This requirement modifies the requirement in section 4 to "keep intact all notices".
- c) You must license the entire work, as a whole, under this License to anyone who comes into possession of a copy. This License will therefore apply, along with any applicable section 7 additional terms, to the whole of the work, and all its parts, regardless of how they are packaged. This License gives no permission to license the work in any other way, but it does not invalidate such permission if you have separately received it.
- d) If the work has interactive user interfaces, each must display Appropriate Legal Notices; however, if the Program has interactive interfaces that do not display Appropriate Legal Notices, your work need not make them do so.

A compilation of a covered work with other separate and independent works, which are not by their nature extensions of the covered work,

and which are not combined with it such as to form a larger program, in or on a volume of a storage or distribution medium, is called an "aggregate" if the compilation and its resulting copyright are not used to limit the access or legal rights of the compilation's users beyond what the individual works permit. Inclusion of a covered work in an aggregate does not cause this License to apply to the other parts of the aggregate.

6. Conveying Non-Source Forms.

You may convey a covered work in object code form under the terms of sections 4 and 5, provided that you also convey the machine-readable Corresponding Source under the terms of this License, in one of these ways:

- a) Convey the object code in, or embodied in, a physical product (including a physical distribution medium), accompanied by the Corresponding Source fixed on a durable physical medium customarily used for software interchange.
- b) Convey the object code in, or embodied in, a physical product (including a physical distribution medium), accompanied by a written offer, valid for at least three years and valid for as long as you offer spare parts or customer support for that product model, to give anyone who possesses the object code either (1) a copy of the Corresponding Source for all the software in the product that is covered by this License, on a durable physical medium customarily used for software interchange, for a price no more than your reasonable cost of physically performing this conveying of source, or (2) access to copy the Corresponding Source from a network server at no charge.
- c) Convey individual copies of the object code with a copy of the written offer to provide the Corresponding Source. This alternative is allowed only occasionally and noncommercially, and only if you received the object code with such an offer, in accord with subsection 6b.
- d) Convey the object code by offering access from a designated place (gratis or for a charge), and offer equivalent access to the Corresponding Source in the same way through the same place at no further charge. You need not require recipients to copy the Corresponding Source along with the object code. If the place to copy the object code is a network server, the Corresponding Source may be on a different server (operated by you or a third party) that supports equivalent copying facilities, provided you maintain clear directions next to the object code saying where to find the Corresponding Source. Regardless of what server hosts the

Corresponding Source, you remain obligated to ensure that it is available for as long as needed to satisfy these requirements.

- e) Convey the object code using peer-to-peer transmission, provided you inform other peers where the object code and Corresponding Source of the work are being offered to the general public at no charge under subsection 6d.

A separable portion of the object code, whose source code is excluded from the Corresponding Source as a System Library, need not be included in conveying the object code work.

A "User Product" is either (1) a "consumer product", which means any tangible personal property which is normally used for personal, family, or household purposes, or (2) anything designed or sold for incorporation into a dwelling. In determining whether a product is a consumer product, doubtful cases shall be resolved in favor of coverage. For a particular product received by a particular user, "normally used" refers to a typical or common use of that class of product, regardless of the status of the particular user or of the way in which the particular user actually uses, or expects or is expected to use, the product. A product is a consumer product regardless of whether the product has substantial commercial, industrial or non-consumer uses, unless such uses represent the only significant mode of use of the product.

"Installation Information" for a User Product means any methods, procedures, authorization keys, or other information required to install and execute modified versions of a covered work in that User Product from a modified version of its Corresponding Source. The information must suffice to ensure that the continued functioning of the modified object code is in no case prevented or interfered with solely because modification has been made.

If you convey an object code work under this section in, or with, or specifically for use in, a User Product, and the conveying occurs as part of a transaction in which the right of possession and use of the User Product is transferred to the recipient in perpetuity or for a fixed term (regardless of how the transaction is characterized), the Corresponding Source conveyed under this section must be accompanied by the Installation Information. But this requirement does not apply if neither you nor any third party retains the ability to install modified object code on the User Product (for example, the work has been installed in ROM).

The requirement to provide Installation Information does not include a requirement to continue to provide support service, warranty, or updates for a work that has been modified or installed by the recipient, or for the User Product in which it has been modified or installed. Access to a

network may be denied when the modification itself materially and adversely affects the operation of the network or violates the rules and protocols for communication across the network.

Corresponding Source conveyed, and Installation Information provided, in accord with this section must be in a format that is publicly documented (and with an implementation available to the public in source code form), and must require no special password or key for unpacking, reading or copying.

7. Additional Terms.

"Additional permissions" are terms that supplement the terms of this License by making exceptions from one or more of its conditions.

Additional permissions that are applicable to the entire Program shall be treated as though they were included in this License, to the extent that they are valid under applicable law. If additional permissions apply only to part of the Program, that part may be used separately under those permissions, but the entire Program remains governed by this License without regard to the additional permissions.

When you convey a copy of a covered work, you may at your option remove any additional permissions from that copy, or from any part of it. (Additional permissions may be written to require their own removal in certain cases when you modify the work.) You may place additional permissions on material, added by you to a covered work, for which you have or can give appropriate copyright permission.

Notwithstanding any other provision of this License, for material you add to a covered work, you may (if authorized by the copyright holders of that material) supplement the terms of this License with terms:

- a) Disclaiming warranty or limiting liability differently from the terms of sections 15 and 16 of this License; or
- b) Requiring preservation of specified reasonable legal notices or author attributions in that material or in the Appropriate Legal Notices displayed by works containing it; or
- c) Prohibiting misrepresentation of the origin of that material, or requiring that modified versions of such material be marked in reasonable ways as different from the original version; or
- d) Limiting the use for publicity purposes of names of licensors or authors of the material; or
- e) Declining to grant rights under trademark law for use of some trade names, trademarks, or service marks; or

f) Requiring indemnification of licensors and authors of that material by anyone who conveys the material (or modified versions of it) with contractual assumptions of liability to the recipient, for any liability that these contractual assumptions directly impose on those licensors and authors.

All other non-permissive additional terms are considered "further restrictions" within the meaning of section 10. If the Program as you received it, or any part of it, contains a notice stating that it is governed by this License along with a term that is a further restriction, you may remove that term. If a license document contains a further restriction but permits relicensing or conveying under this License, you may add to a covered work material governed by the terms of that license document, provided that the further restriction does not survive such relicensing or conveying.

If you add terms to a covered work in accord with this section, you must place, in the relevant source files, a statement of the additional terms that apply to those files, or a notice indicating where to find the applicable terms.

Additional terms, permissive or non-permissive, may be stated in the form of a separately written license, or stated as exceptions; the above requirements apply either way.

8. Termination.

You may not propagate or modify a covered work except as expressly provided under this License. Any attempt otherwise to propagate or modify it is void, and will automatically terminate your rights under this License (including any patent licenses granted under the third paragraph of section 11).

However, if you cease all violation of this License, then your license from a particular copyright holder is reinstated (a) provisionally, unless and until the copyright holder explicitly and finally terminates your license, and (b) permanently, if the copyright holder fails to notify you of the violation by some reasonable means prior to 60 days after the cessation.

Moreover, your license from a particular copyright holder is reinstated permanently if the copyright holder notifies you of the violation by some reasonable means, this is the first time you have received notice of violation of this License (for any work) from that copyright holder, and you cure the violation prior to 30 days after your receipt of the notice.

Termination of your rights under this section does not terminate the licenses of parties who have received copies or rights from you under this License. If your rights have been terminated and not permanently reinstated, you do not qualify to receive new licenses for the same material under section 10.

9. Acceptance Not Required for Having Copies.

You are not required to accept this License in order to receive or run a copy of the Program. Ancillary propagation of a covered work occurring solely as a consequence of using peer-to-peer transmission to receive a copy likewise does not require acceptance. However, nothing other than this License grants you permission to propagate or modify any covered work. These actions infringe copyright if you do not accept this License. Therefore, by modifying or propagating a covered work, you indicate your acceptance of this License to do so.

10. Automatic Licensing of Downstream Recipients.

Each time you convey a covered work, the recipient automatically receives a license from the original licensors, to run, modify and propagate that work, subject to this License. You are not responsible for enforcing compliance by third parties with this License.

An "entity transaction" is a transaction transferring control of an organization, or substantially all assets of one, or subdividing an organization, or merging organizations. If propagation of a covered work results from an entity transaction, each party to that transaction who receives a copy of the work also receives whatever licenses to the work the party's predecessor in interest had or could give under the previous paragraph, plus a right to possession of the Corresponding Source of the work from the predecessor in interest, if the predecessor has it or can get it with reasonable efforts.

You may not impose any further restrictions on the exercise of the rights granted or affirmed under this License. For example, you may not impose a license fee, royalty, or other charge for exercise of rights granted under this License, and you may not initiate litigation (including a cross-claim or counterclaim in a lawsuit) alleging that any patent claim is infringed by making, using, selling, offering for sale, or importing the Program or any portion of it.

11. Patents.

A "contributor" is a copyright holder who authorizes use under this License of the Program or a work on which the Program is based. The work thus licensed is called the contributor's "contributor version".

A contributor's "essential patent claims" are all patent claims owned or controlled by the contributor, whether already acquired or hereafter acquired, that would be infringed by some manner, permitted by this License, of making, using, or selling its contributor version, but do not include claims that would be infringed only as a consequence of further modification of the contributor version. For purposes of this definition, "control" includes the right to grant patent sublicenses in a manner consistent with the requirements of this License.

Each contributor grants you a non-exclusive, worldwide, royalty-free patent license under the contributor's essential patent claims, to make, use, sell, offer for sale, import and otherwise run, modify and propagate the contents of its contributor version.

In the following three paragraphs, a "patent license" is any express agreement or commitment, however denominated, not to enforce a patent (such as an express permission to practice a patent or covenant not to sue for patent infringement). To "grant" such a patent license to a party means to make such an agreement or commitment not to enforce a patent against the party.

If you convey a covered work, knowingly relying on a patent license, and the Corresponding Source of the work is not available for anyone to copy, free of charge and under the terms of this License, through a publicly available network server or other readily accessible means, then you must either (1) cause the Corresponding Source to be so available, or (2) arrange to deprive yourself of the benefit of the patent license for this particular work, or (3) arrange, in a manner consistent with the requirements of this License, to extend the patent license to downstream recipients. "Knowingly relying" means you have actual knowledge that, but for the patent license, your conveying the covered work in a country, or your recipient's use of the covered work in a country, would infringe one or more identifiable patents in that country that you have reason to believe are valid.

If, pursuant to or in connection with a single transaction or arrangement, you convey, or propagate by procuring conveyance of, a covered work, and grant a patent license to some of the parties receiving the covered work authorizing them to use, propagate, modify or convey a specific copy of the covered work, then the patent license you grant is automatically extended to all recipients of the covered work and works based on it.

A patent license is "discriminatory" if it does not include within the scope of its coverage, prohibits the exercise of, or is conditioned on the non-exercise of one or more of the rights that are specifically granted under this License. You may not convey a covered

work if you are a party to an arrangement with a third party that is in the business of distributing software, under which you make payment to the third party based on the extent of your activity of conveying the work, and under which the third party grants, to any of the parties who would receive the covered work from you, a discriminatory patent license (a) in connection with copies of the covered work conveyed by you (or copies made from those copies), or (b) primarily for and in connection with specific products or compilations that contain the covered work, unless you entered into that arrangement, or that patent license was granted, prior to 28 March 2007.

Nothing in this License shall be construed as excluding or limiting any implied license or other defenses to infringement that may otherwise be available to you under applicable patent law.

12. No Surrender of Others' Freedom.

If conditions are imposed on you (whether by court order, agreement or otherwise) that contradict the conditions of this License, they do not excuse you from the conditions of this License. If you cannot convey a covered work so as to satisfy simultaneously your obligations under this License and any other pertinent obligations, then as a consequence you may not convey it at all. For example, if you agree to terms that obligate you to collect a royalty for further conveying from those to whom you convey the Program, the only way you could satisfy both those terms and this License would be to refrain entirely from conveying the Program.

13. Use with the GNU Affero General Public License.

Notwithstanding any other provision of this License, you have permission to link or combine any covered work with a work licensed under version 3 of the GNU Affero General Public License into a single combined work, and to convey the resulting work. The terms of this License will continue to apply to the part which is the covered work, but the special requirements of the GNU Affero General Public License, section 13, concerning interaction through a network will apply to the combination as such.

14. Revised Versions of this License.

The Free Software Foundation may publish revised and/or new versions of the GNU General Public License from time to time. Such new versions will be similar in spirit to the present version, but may differ in detail to address new problems or concerns.

Each version is given a distinguishing version number. If the Program specifies that a certain numbered version of the GNU General Public License "or any later version" applies to it, you have the

option of following the terms and conditions either of that numbered version or of any later version published by the Free Software Foundation. If the Program does not specify a version number of the GNU General Public License, you may choose any version ever published by the Free Software Foundation.

If the Program specifies that a proxy can decide which future versions of the GNU General Public License can be used, that proxy's public statement of acceptance of a version permanently authorizes you to choose that version for the Program.

Later license versions may give you additional or different permissions. However, no additional obligations are imposed on any author or copyright holder as a result of your choosing to follow a later version.

15. Disclaimer of Warranty.

THERE IS NO WARRANTY FOR THE PROGRAM, TO THE EXTENT PERMITTED BY APPLICABLE LAW. EXCEPT WHEN OTHERWISE STATED IN WRITING THE COPYRIGHT HOLDERS AND/OR OTHER PARTIES PROVIDE THE PROGRAM "AS IS" WITHOUT WARRANTY OF ANY KIND, EITHER EXPRESSED OR IMPLIED, INCLUDING, BUT NOT LIMITED TO, THE IMPLIED WARRANTIES OF MERCHANTABILITY AND FITNESS FOR A PARTICULAR PURPOSE. THE ENTIRE RISK AS TO THE QUALITY AND PERFORMANCE OF THE PROGRAM IS WITH YOU. SHOULD THE PROGRAM PROVE DEFECTIVE, YOU ASSUME THE COST OF ALL NECESSARY SERVICING, REPAIR OR CORRECTION.

16. Limitation of Liability.

IN NO EVENT UNLESS REQUIRED BY APPLICABLE LAW OR AGREED TO IN WRITING WILL ANY COPYRIGHT HOLDER, OR ANY OTHER PARTY WHO MODIFIES AND/OR CONVEYS THE PROGRAM AS PERMITTED ABOVE, BE LIABLE TO YOU FOR DAMAGES, INCLUDING ANY GENERAL, SPECIAL, INCIDENTAL OR CONSEQUENTIAL DAMAGES ARISING OUT OF THE USE OR INABILITY TO USE THE PROGRAM (INCLUDING BUT NOT LIMITED TO LOSS OF DATA OR DATA BEING RENDERED INACCURATE OR LOSSES SUSTAINED BY YOU OR THIRD PARTIES OR A FAILURE OF THE PROGRAM TO OPERATE WITH ANY OTHER PROGRAMS), EVEN IF SUCH HOLDER OR OTHER PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES.

17. Interpretation of Sections 15 and 16.

If the disclaimer of warranty and limitation of liability provided above cannot be given local legal effect according to their terms, reviewing courts shall apply local law that most closely approximates an absolute waiver of all civil liability in connection with the Program, unless a warranty or assumption of liability accompanies a copy of the Program in return for a fee.

