# MiA User Guide

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### 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

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*

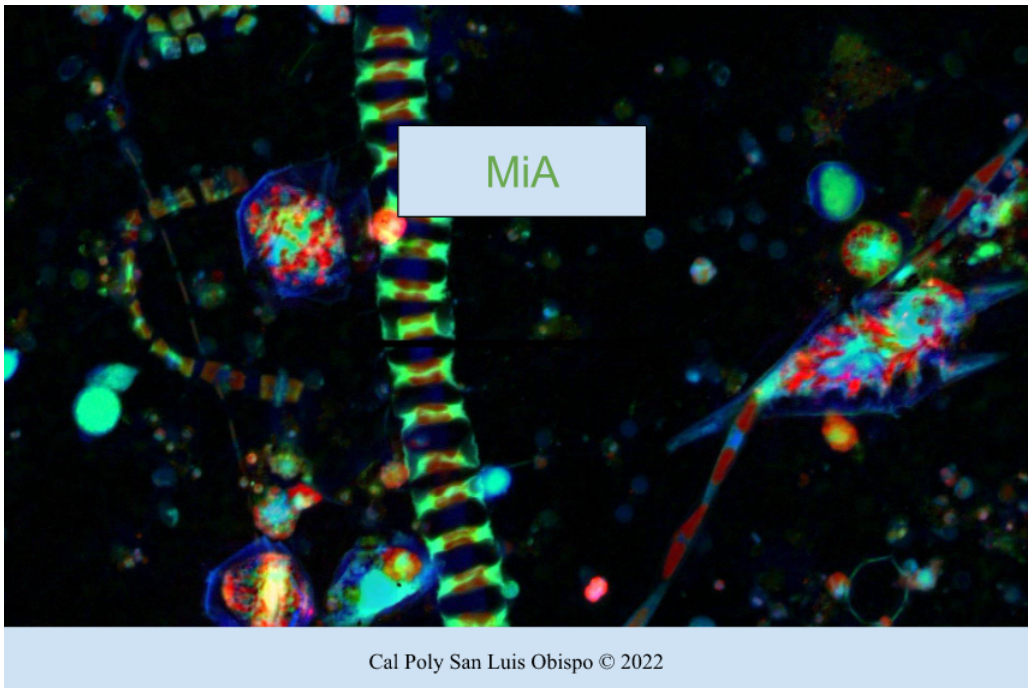



Figure 1: Windows MiA Splash Screen, 2022 edition

*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.

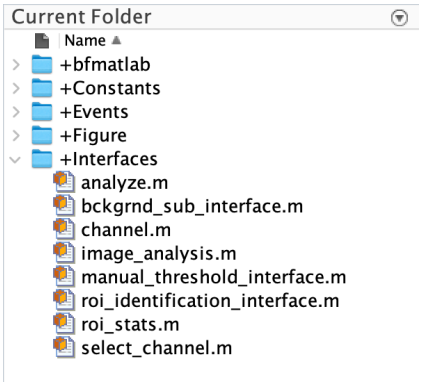



Figure 2: Working Directory

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.

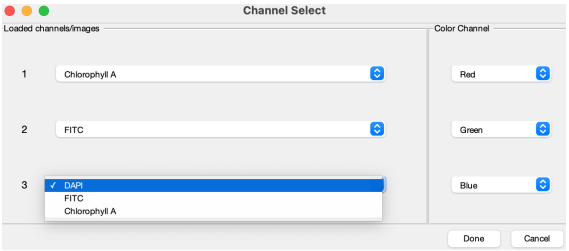



Figure 4: Channel Select Pop-up

****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.

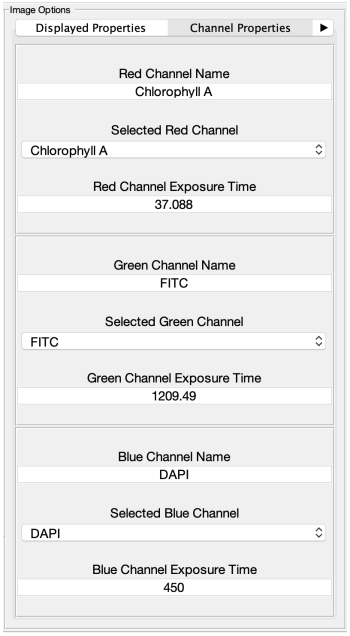



Figure 5: Example Channel Properties Tab

## 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.

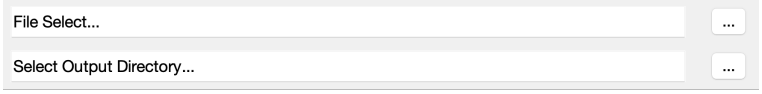



Figure 6: File-Directory Selection Snapshot

## 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).*

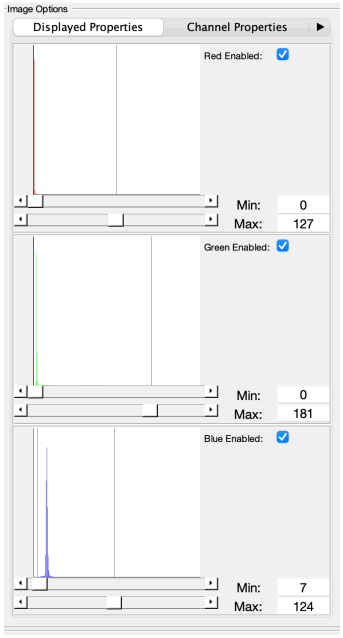



Figure 7: Contrast/Channel Display Panel

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'.

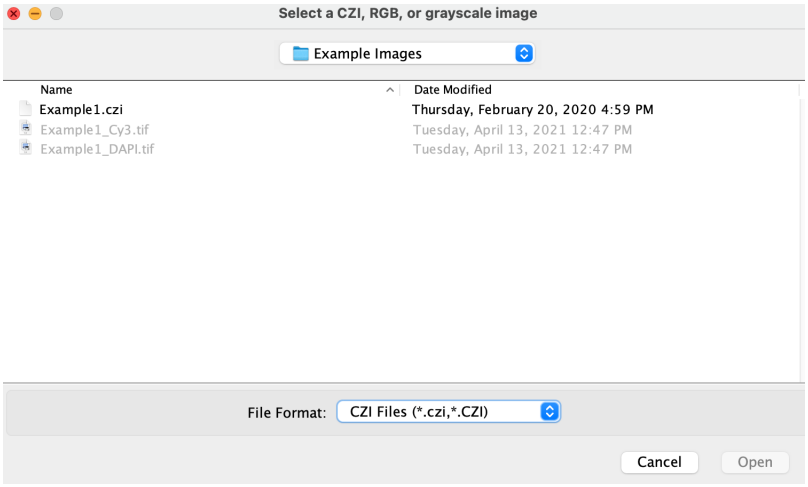



Figure 8: Example 'Load Image(s)' Dialog

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.

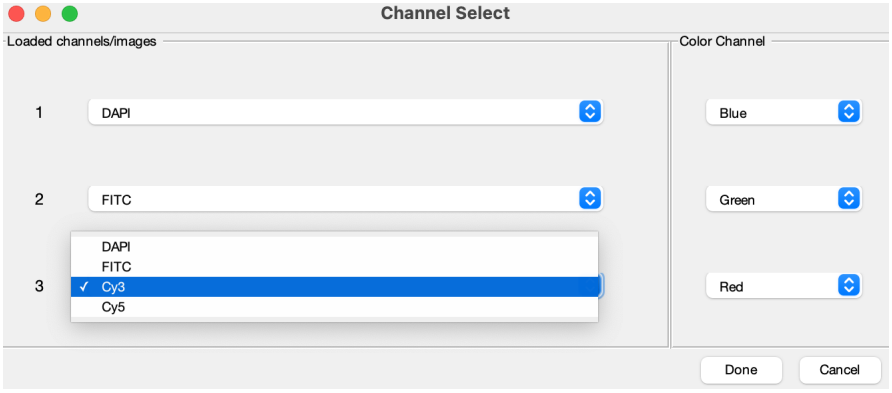



Figure 9: Example 'Channel Select' Dialog: Four-Channel CZI

### 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

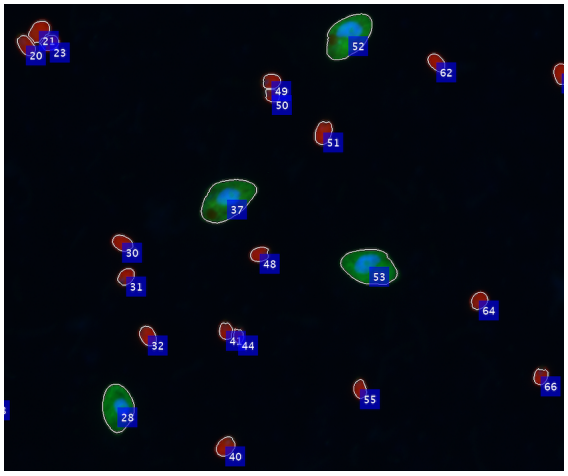
'Save ROI Mask' saves only the current existing ROI binary mask under either the image name or a user-defined name. If any ROIs have been identified as described in Section 4.2.17, the ROI IDs are saved in the created .mat file as well. Upon initially selecting this option, the user will be prompted 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

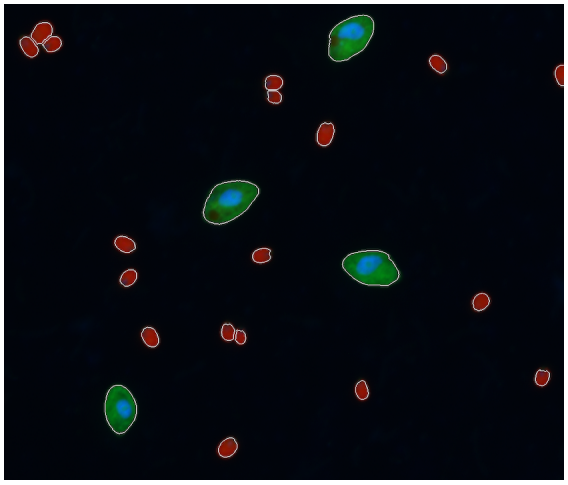
'Save Outlined Cells Image' saves only the 'Outlined Cells' image defined in Figure 10. This image is an exact snapshot of the current displayed setup, including any changed channel contrasts, with any ROI outlines. This image can be useful for sharing current analyses or using in presentations.

#### 4.1.2.6 Save ROI IDs

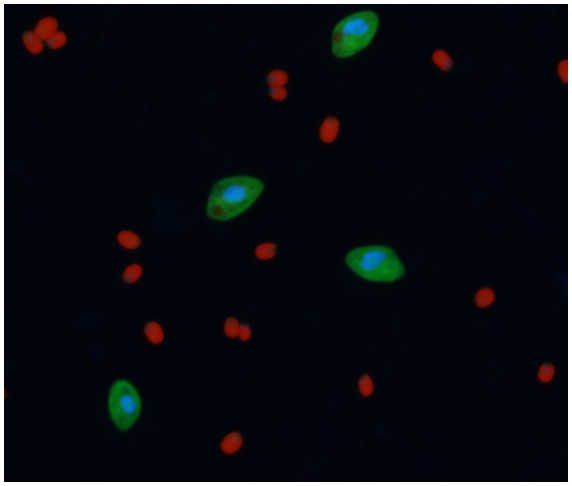
'Save ROI IDs' only becomes enabled once at least one ROI has been identified. Once it's been enabled, you can select this file menu option to save all ROI IDs as a .csv file in the selected output directory. All ROI IDs, regardless of whether they have been identified or not, are included in the export. 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.

4. 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.

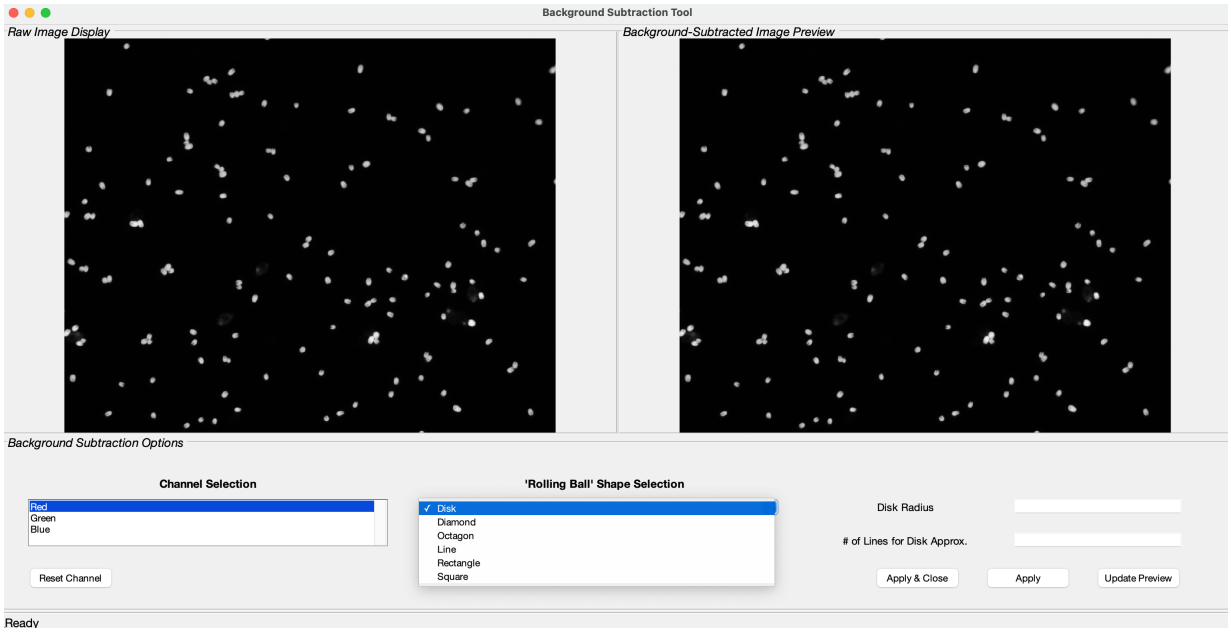



Figure 12: Background Subtraction: Example Window with Rolling Ball Dropdown

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

1. **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.

2. **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.

3. **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.

4. **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.

5. **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

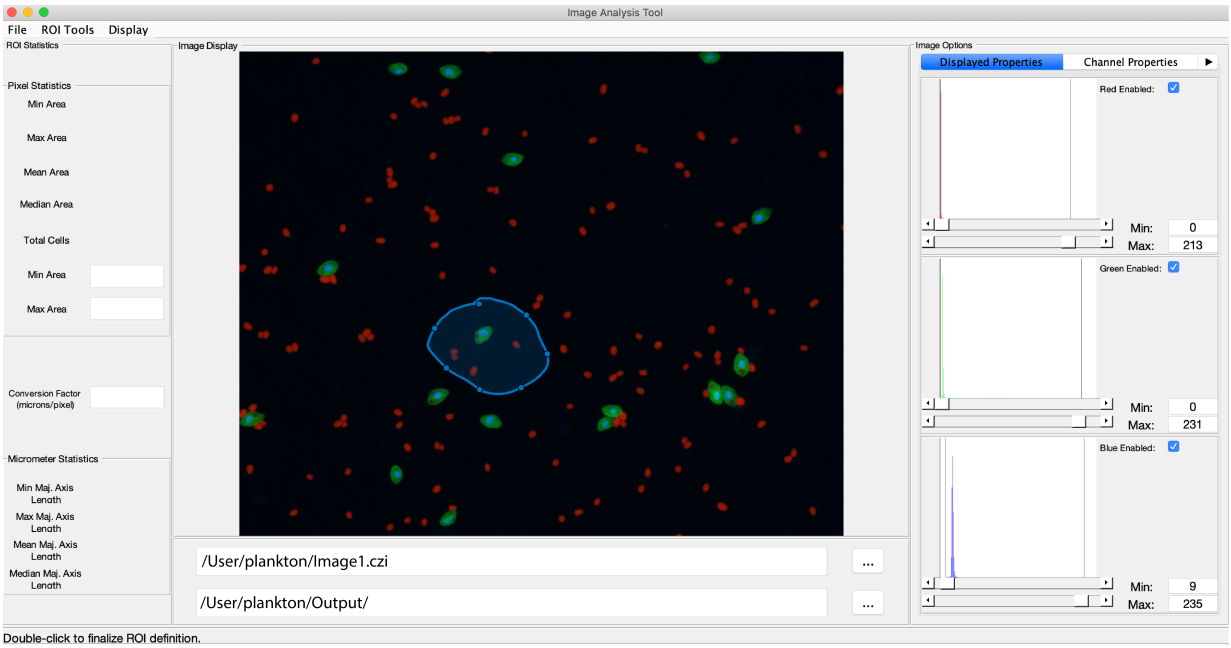



Figure 13: Auto-Threshold ROI: Defining Region

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'.

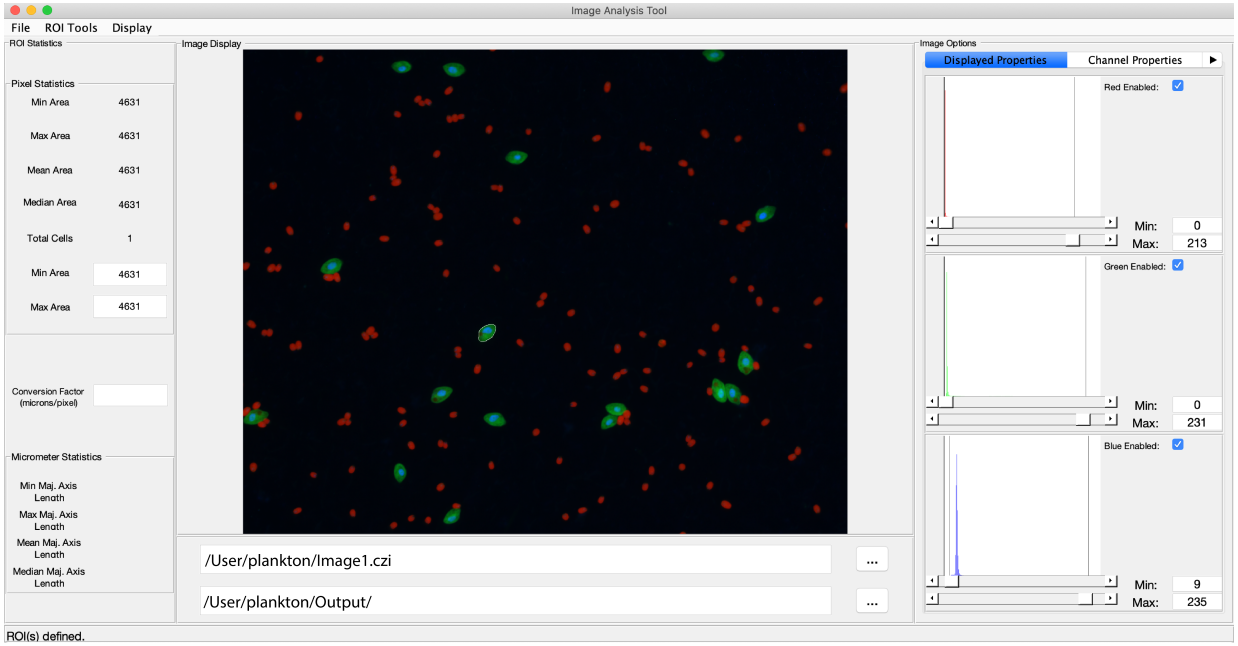



Figure 14: Auto-Threshold ROI: ROI Defined

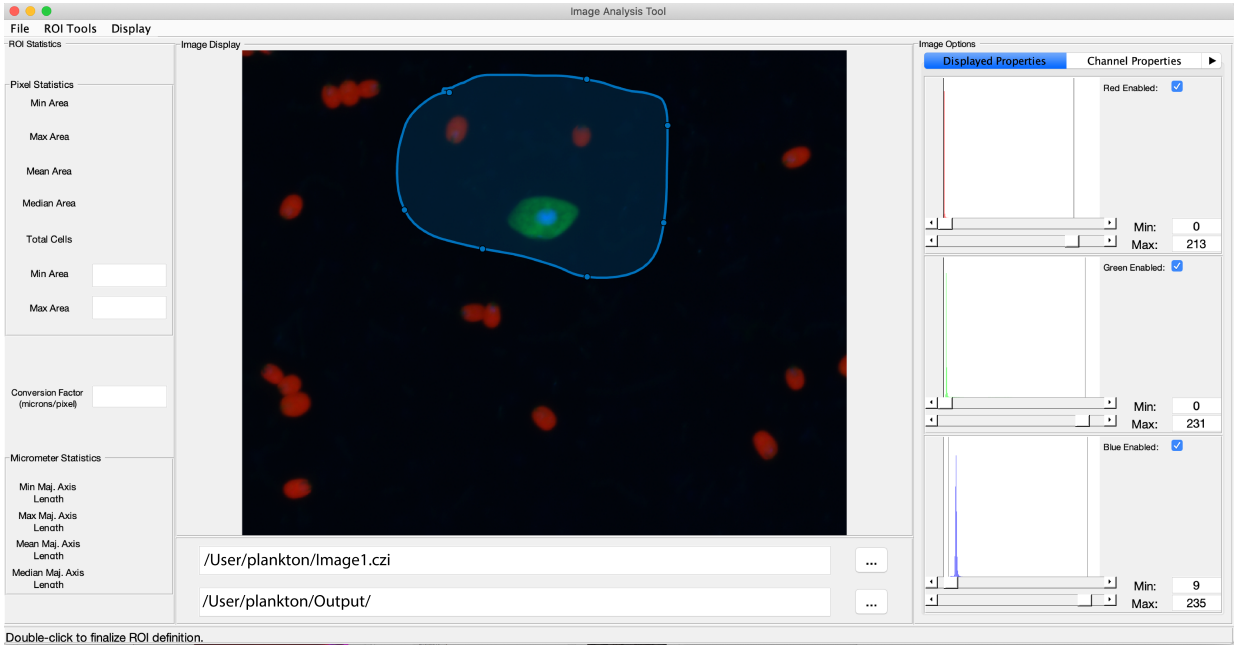



Figure 15: Manual-Threshold ROI: Defining Region

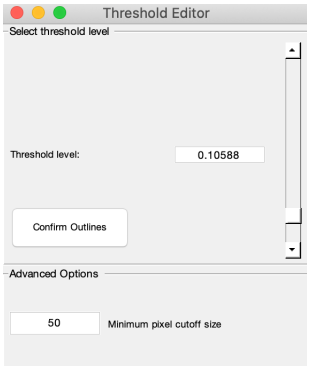



Figure 16: Manual-Threshold ROI: Manual Threshold Interface

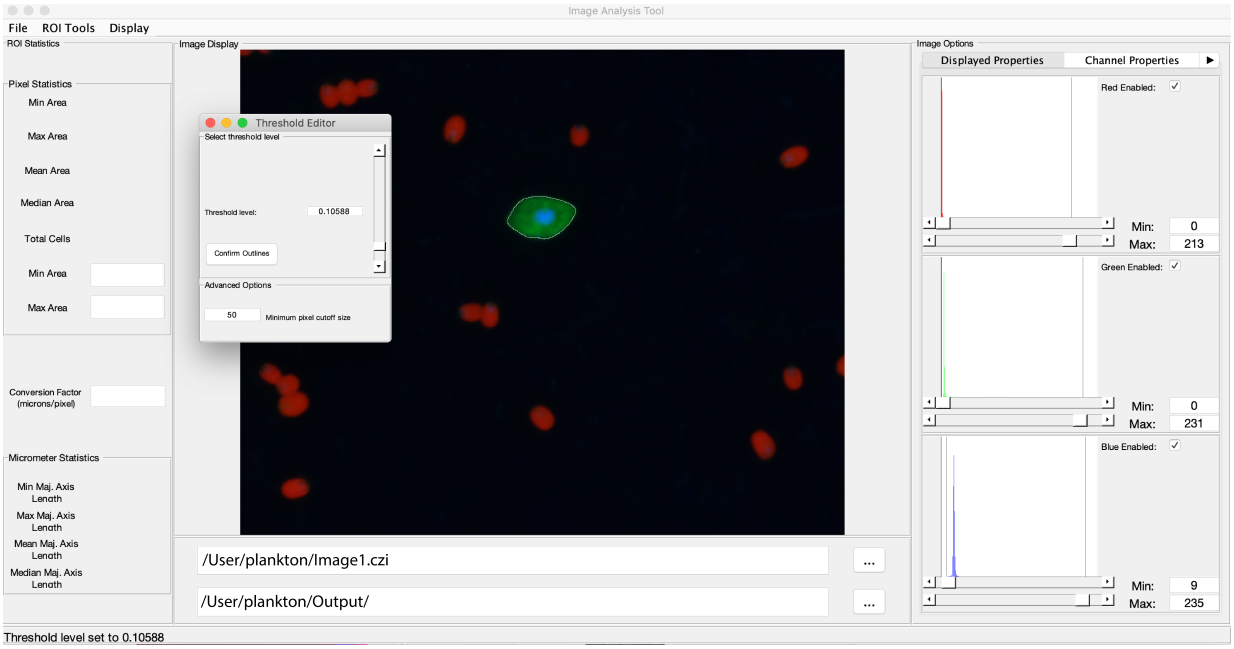



Figure 17: Manual-Threshold ROI: ROI Initial Threshold

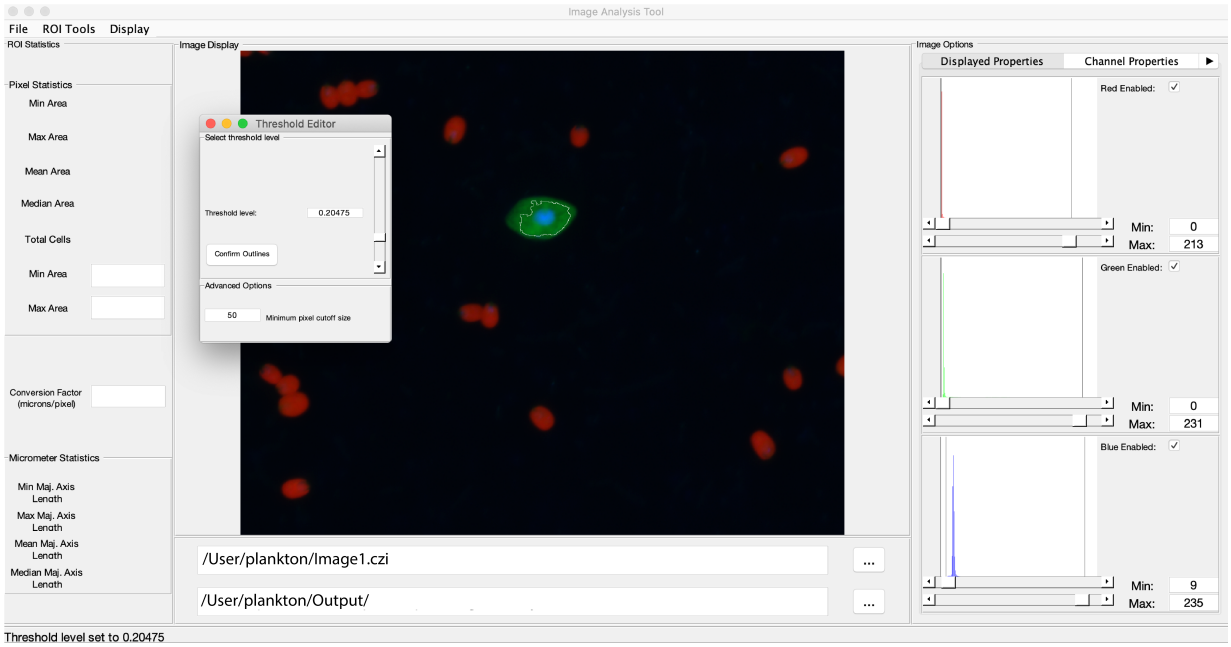



Figure 18: Manual-Threshold ROI: ROI Adjusted Threshold

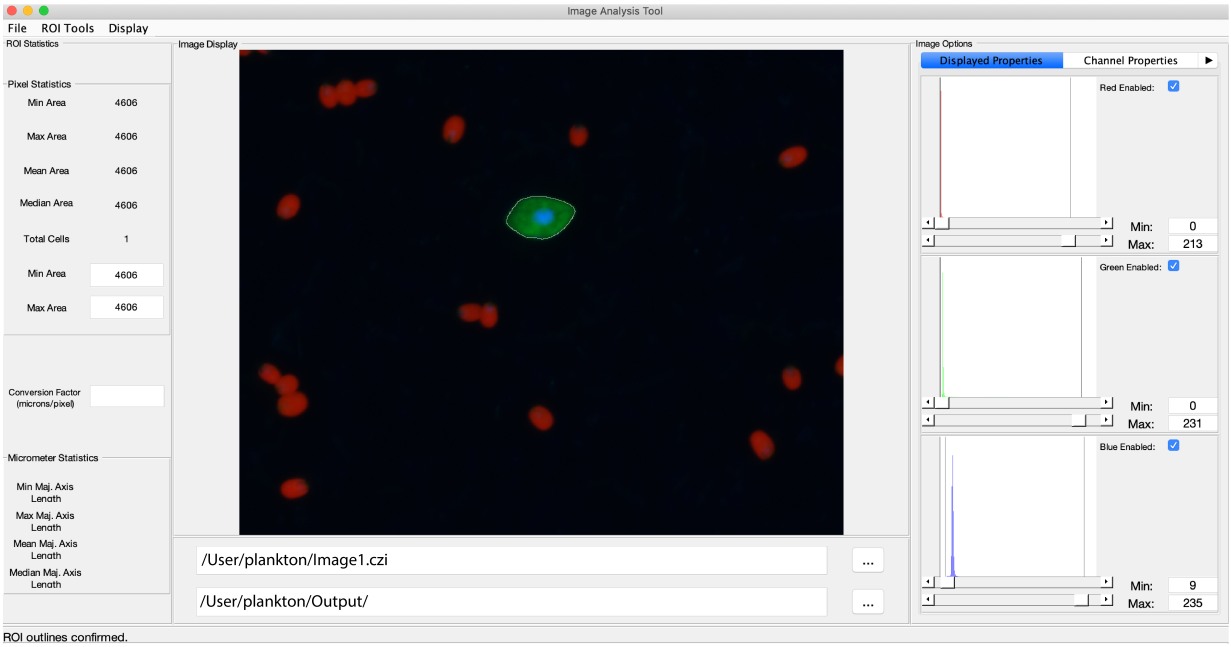



Figure 19: Manual-Threshold ROI: ROI Defined

### 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'.

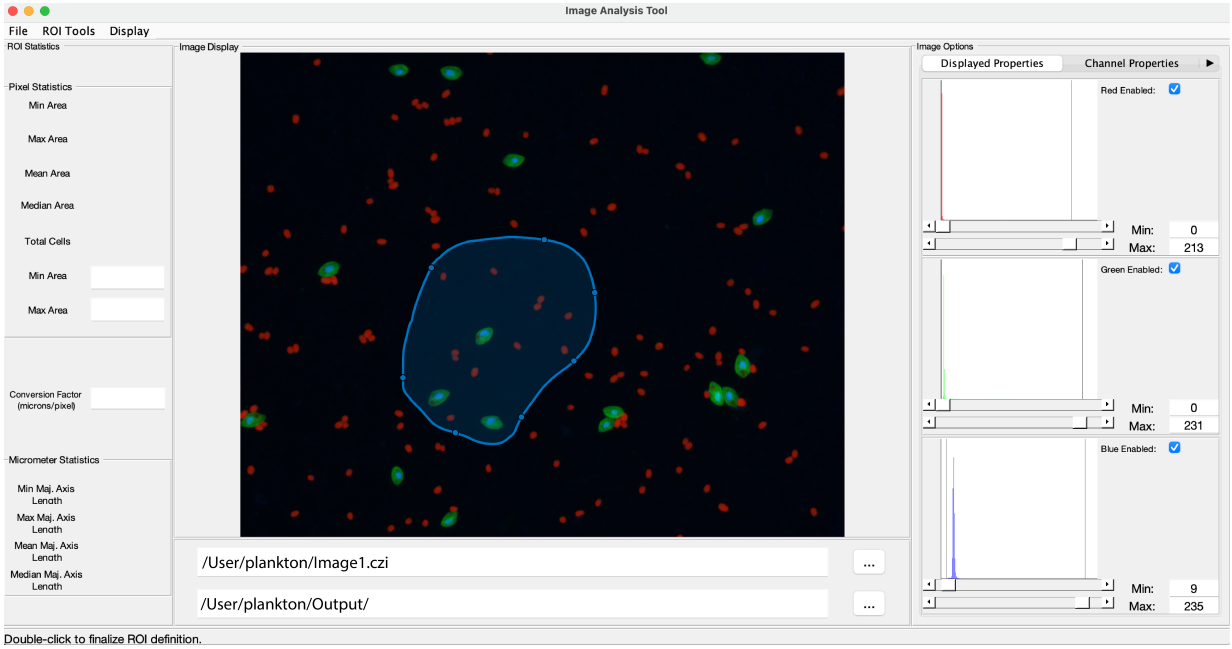



Figure 20: Manual-Threshold Region of ROIs: Defining Region

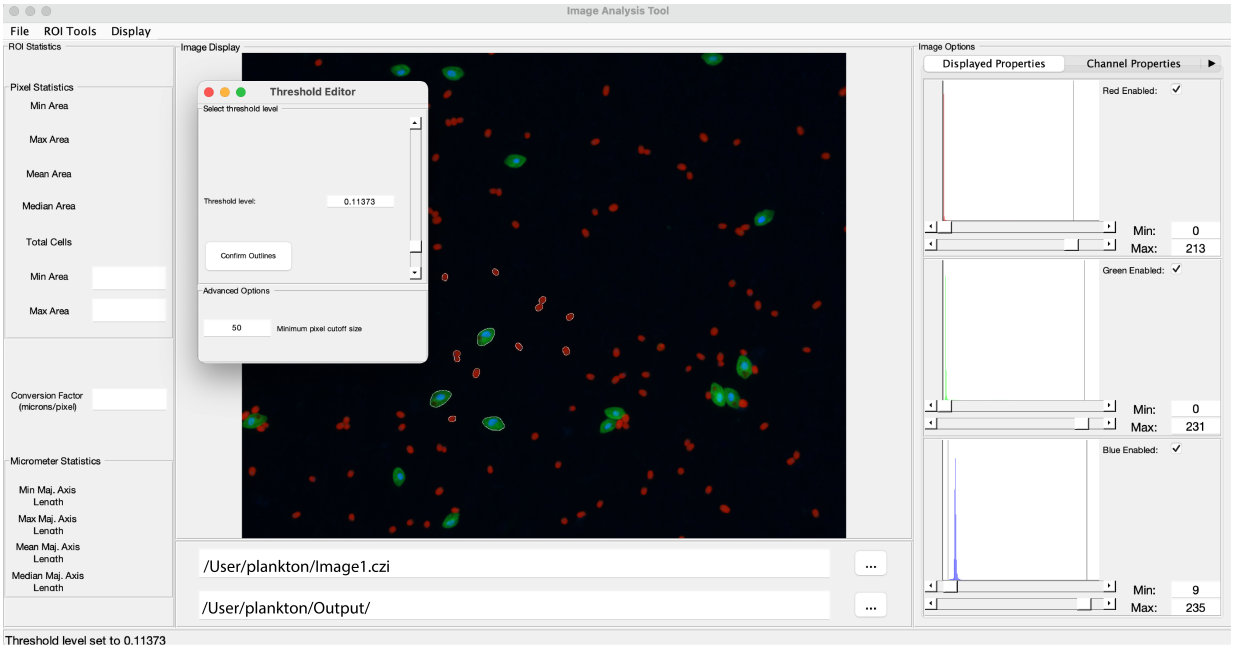



Figure 21: Manual-Threshold Region of ROIs: ROI Initial Threshold

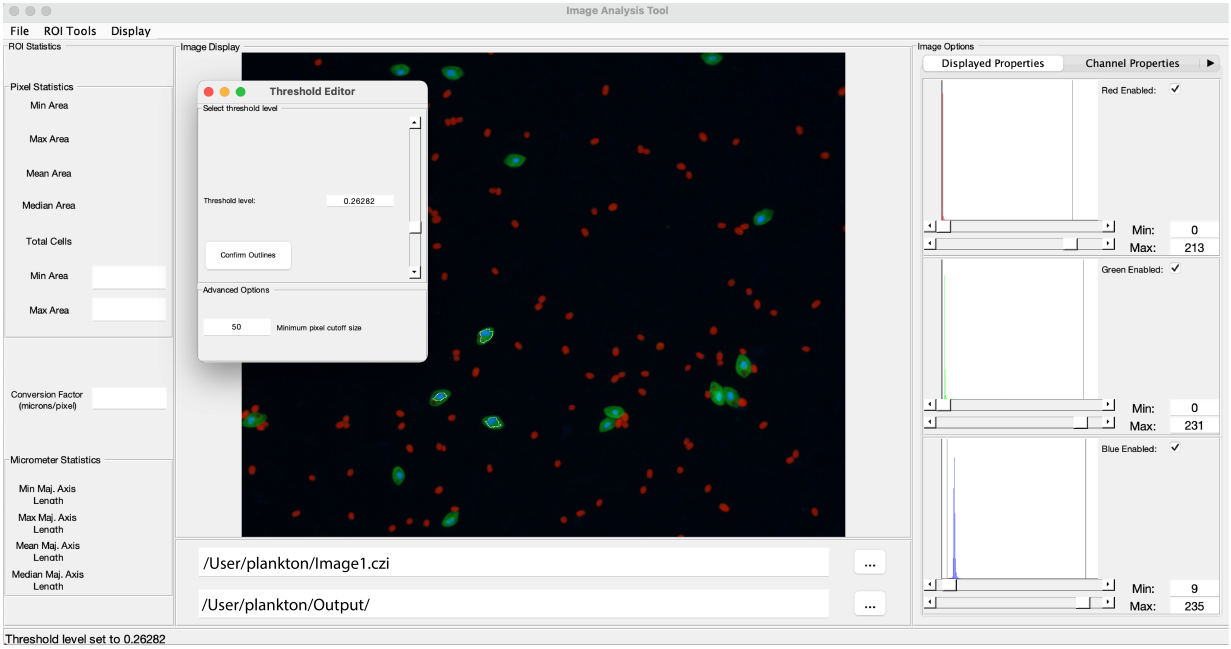



Figure 22: Manual-Threshold Region of ROIs: ROI Adjusted Threshold

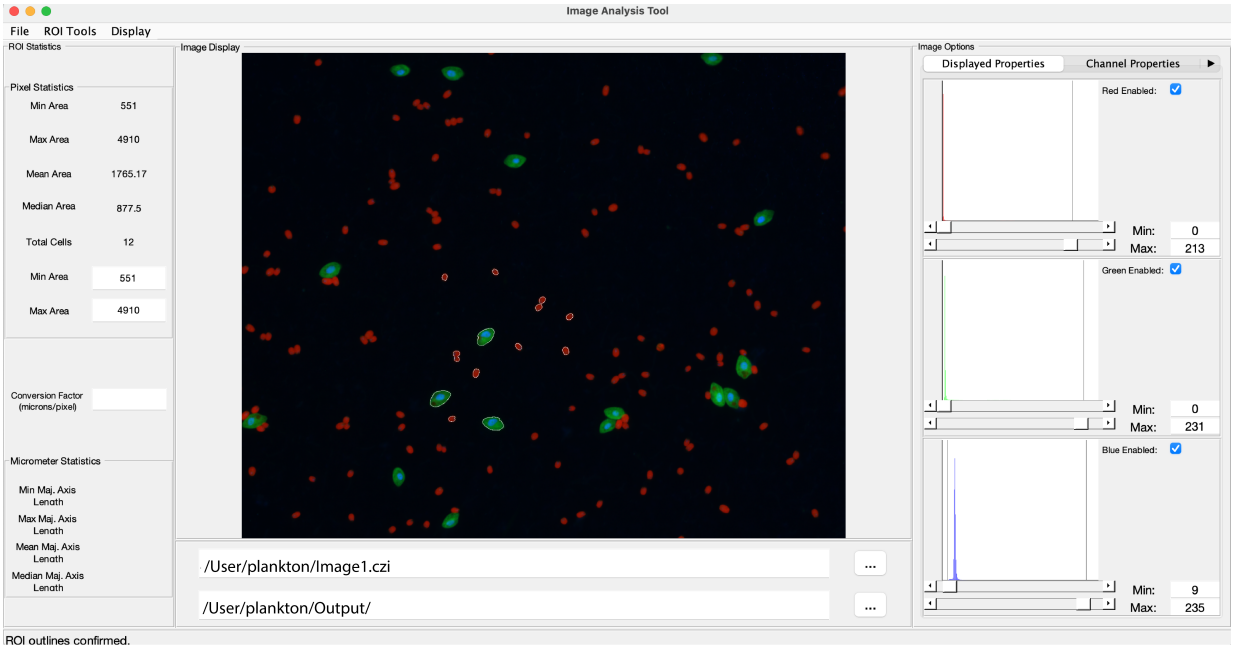



Figure 23: Manual-Threshold Region of ROIs: ROIs Defined

### 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'.

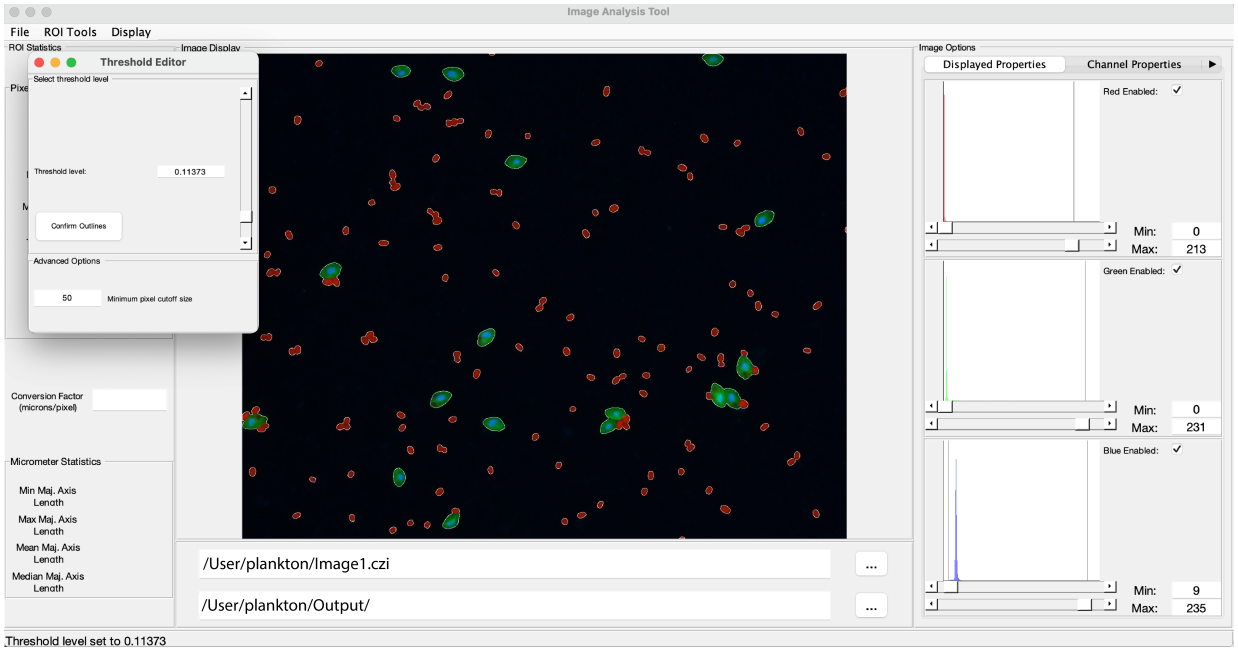



Figure 24: Manual-Threshold All ROIs: ROI Initial Threshold

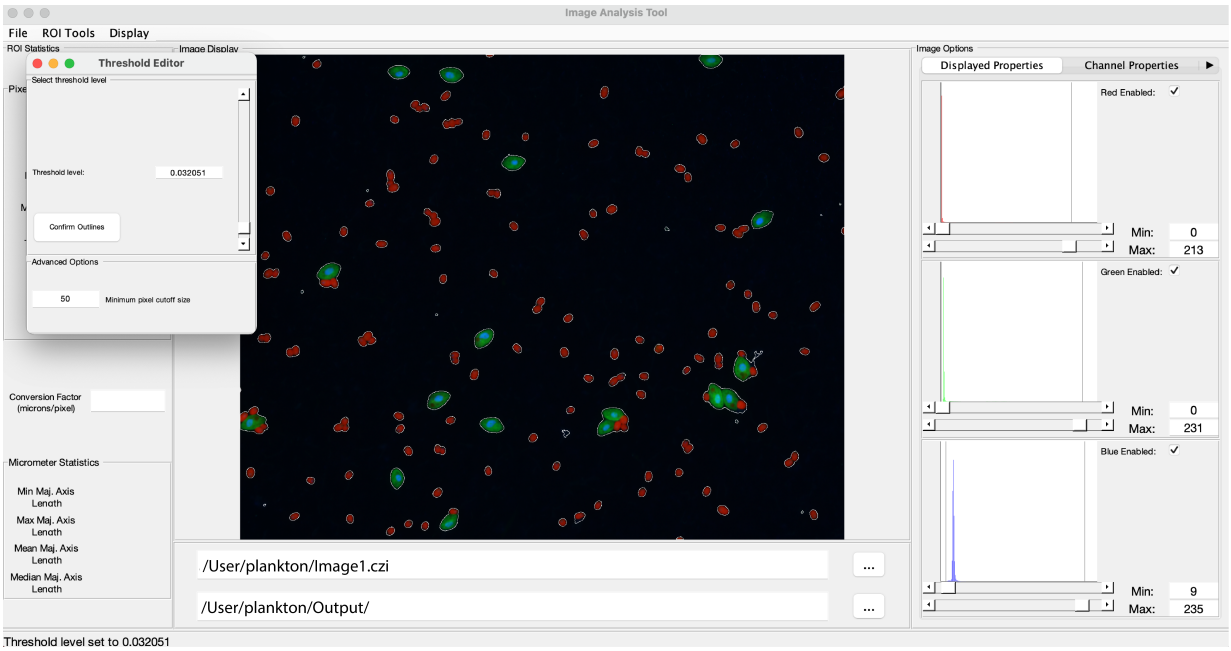



Figure 25: Manual-Threshold All ROIs: ROI Adjusted Threshold

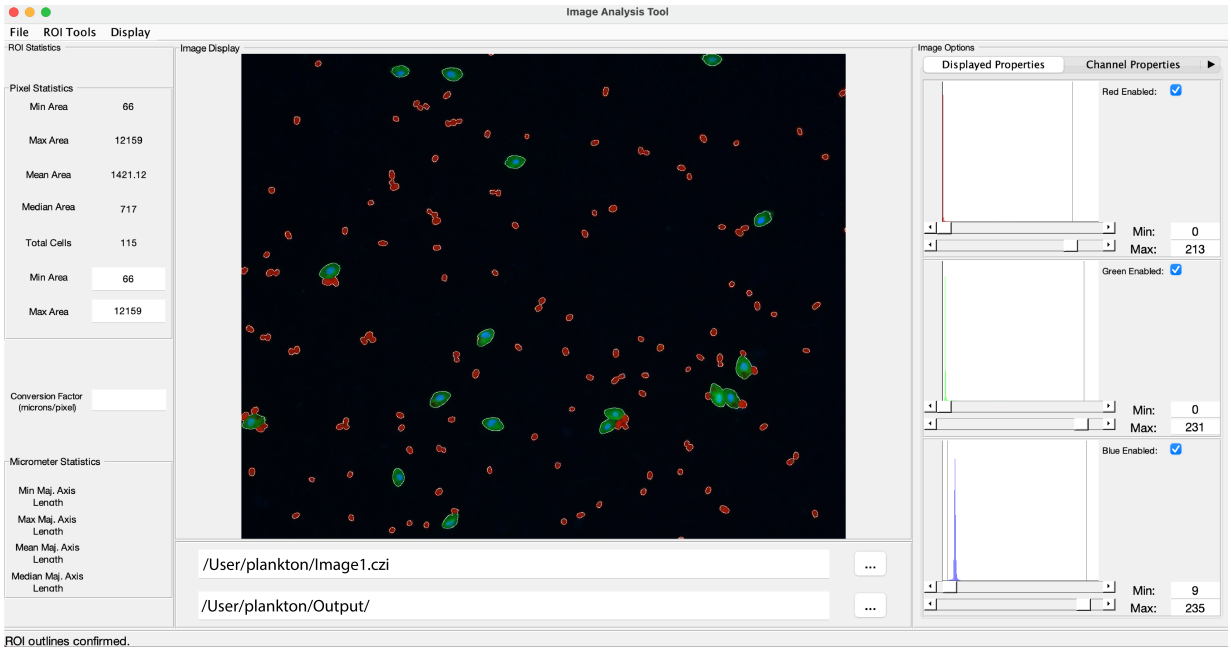



Figure 26: Manual-Threshold All ROIs: ROIs Defined

### 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.

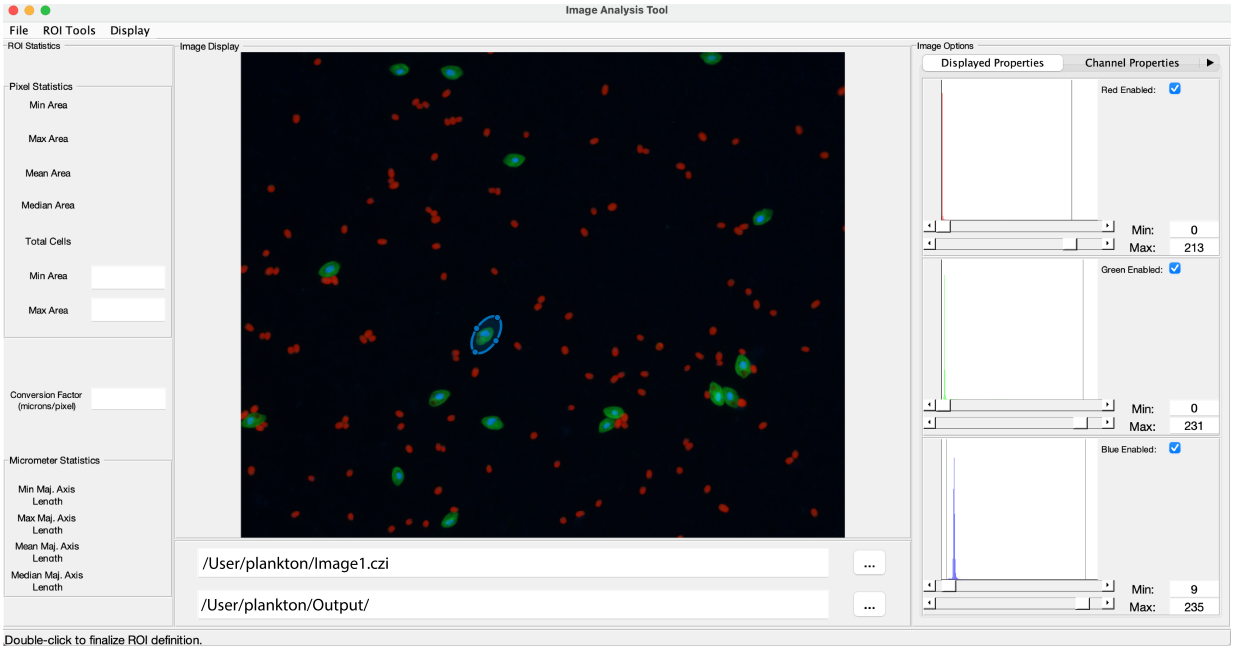



Figure 27: Defining an Ellipse

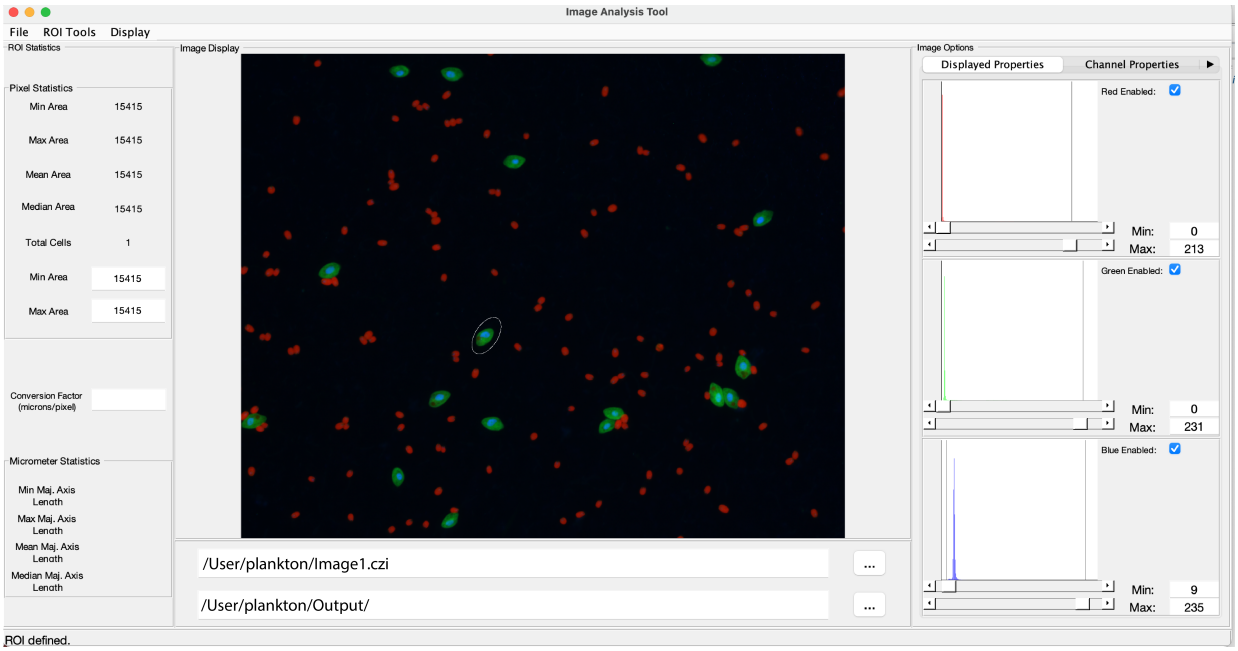



Figure 28: Ellipse Defined

### 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.

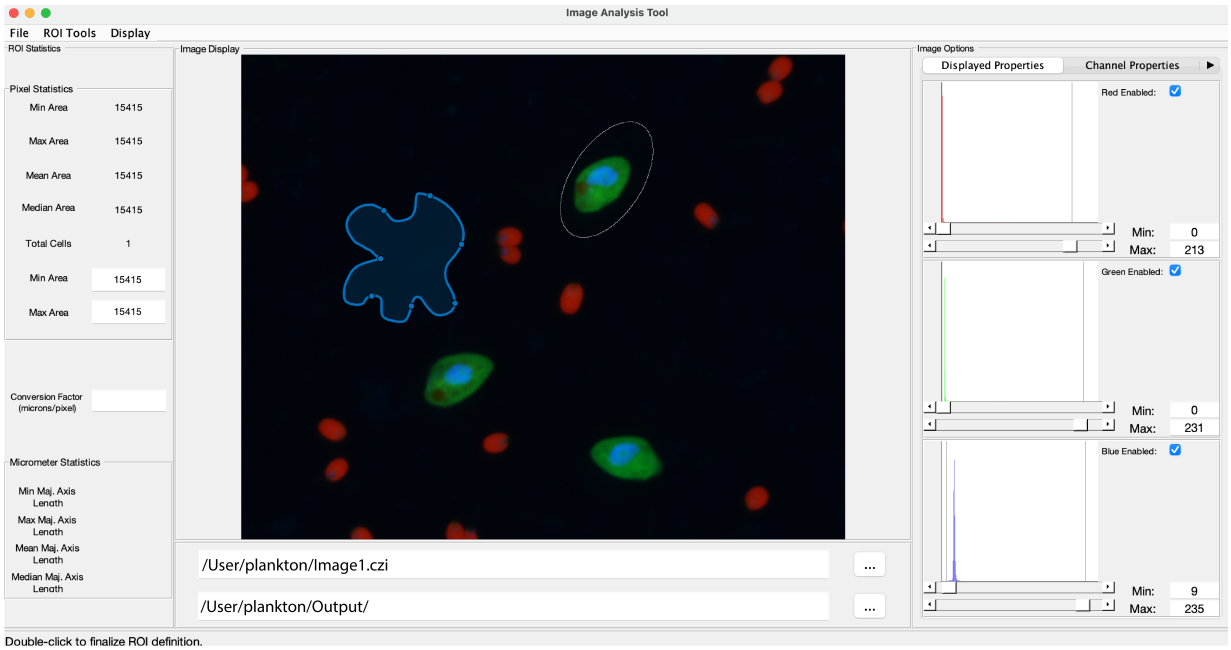



Figure 29: Defining a Freehand

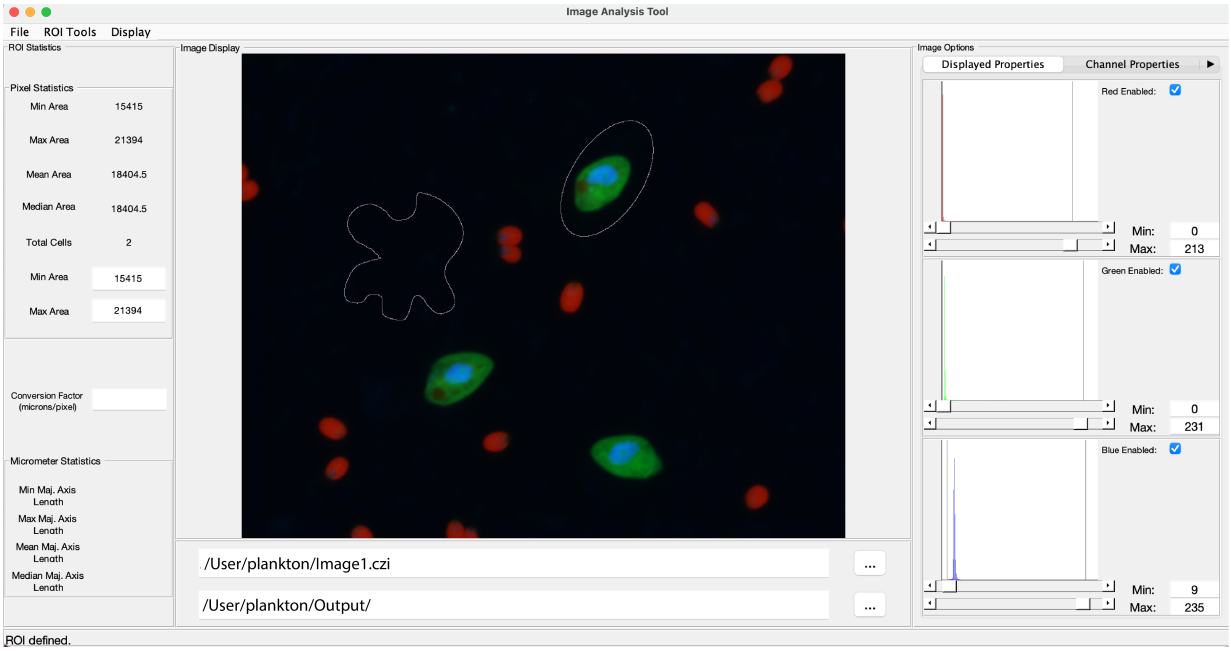



Figure 30: Freehand Defined

### 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.

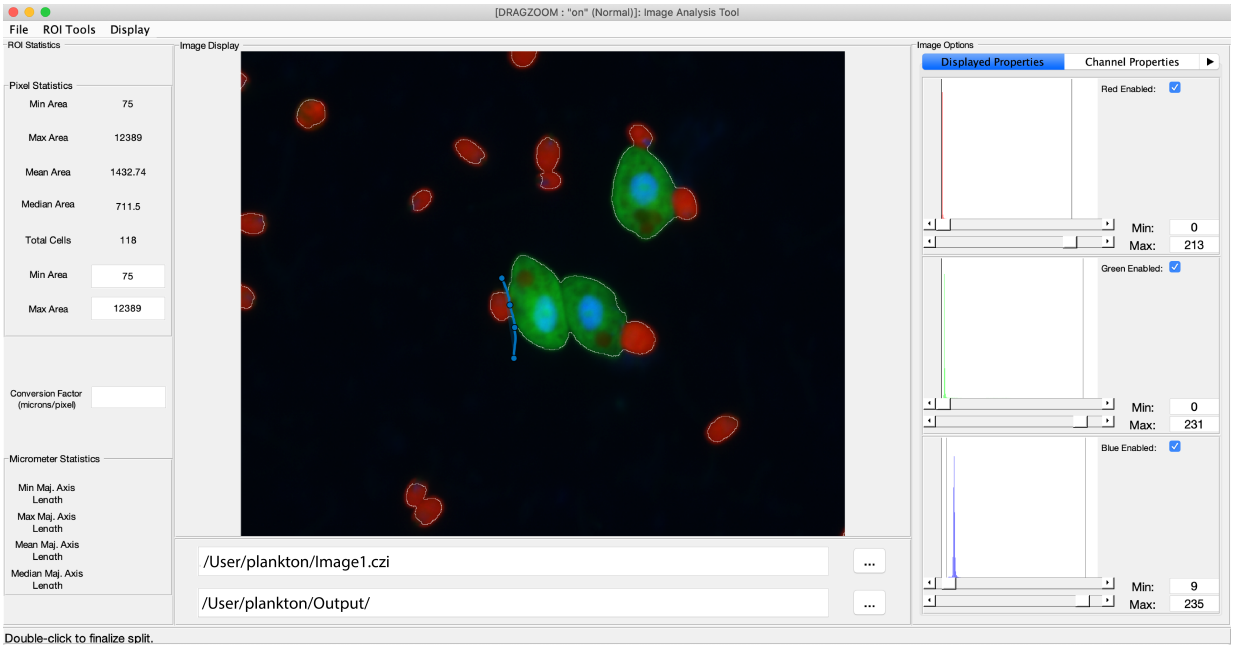



Figure 31: Splitting an ROI

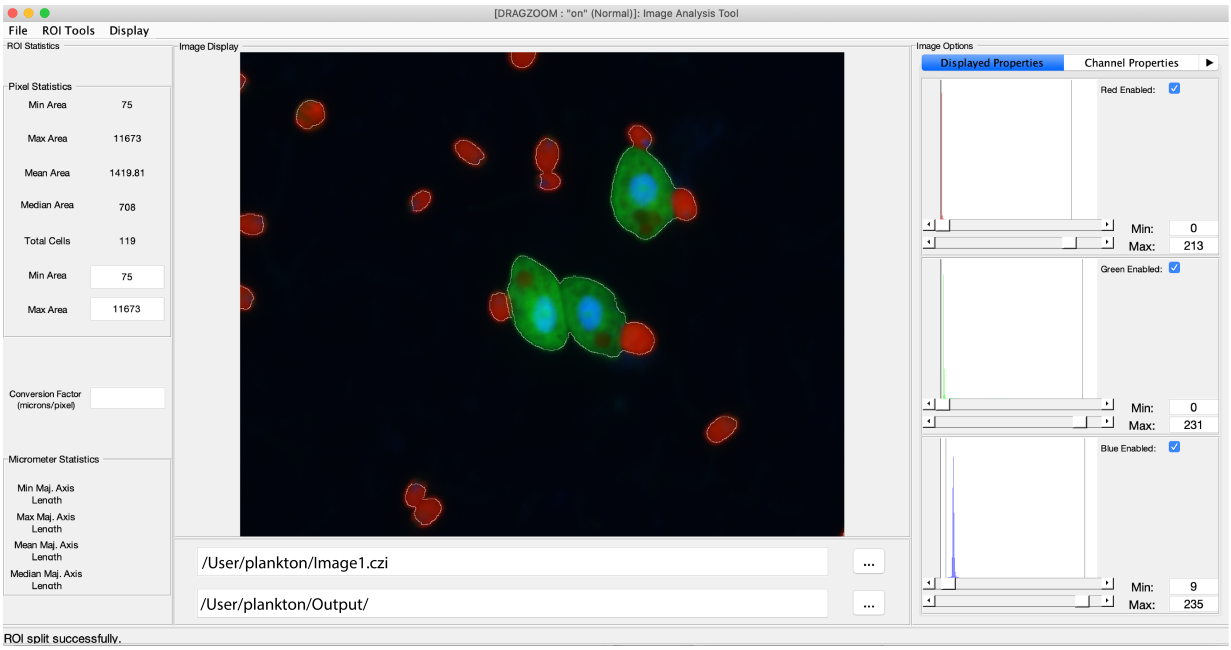



Figure 32: Split Finalized

### 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.

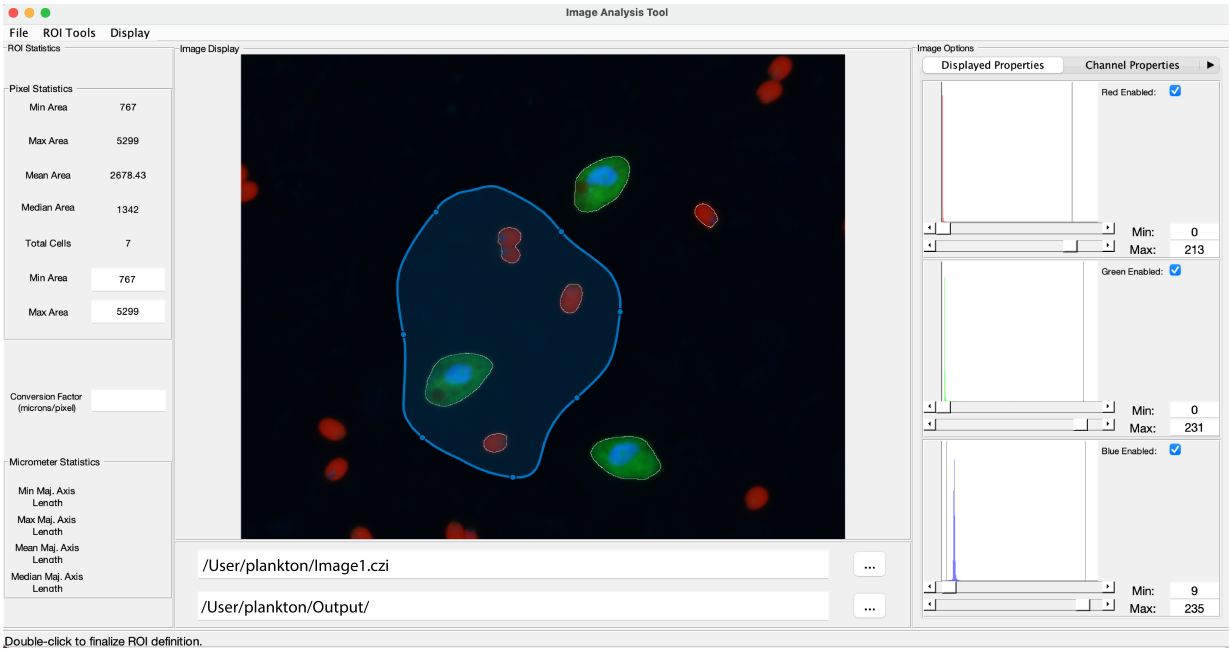



Figure 33: Define Deletion Region

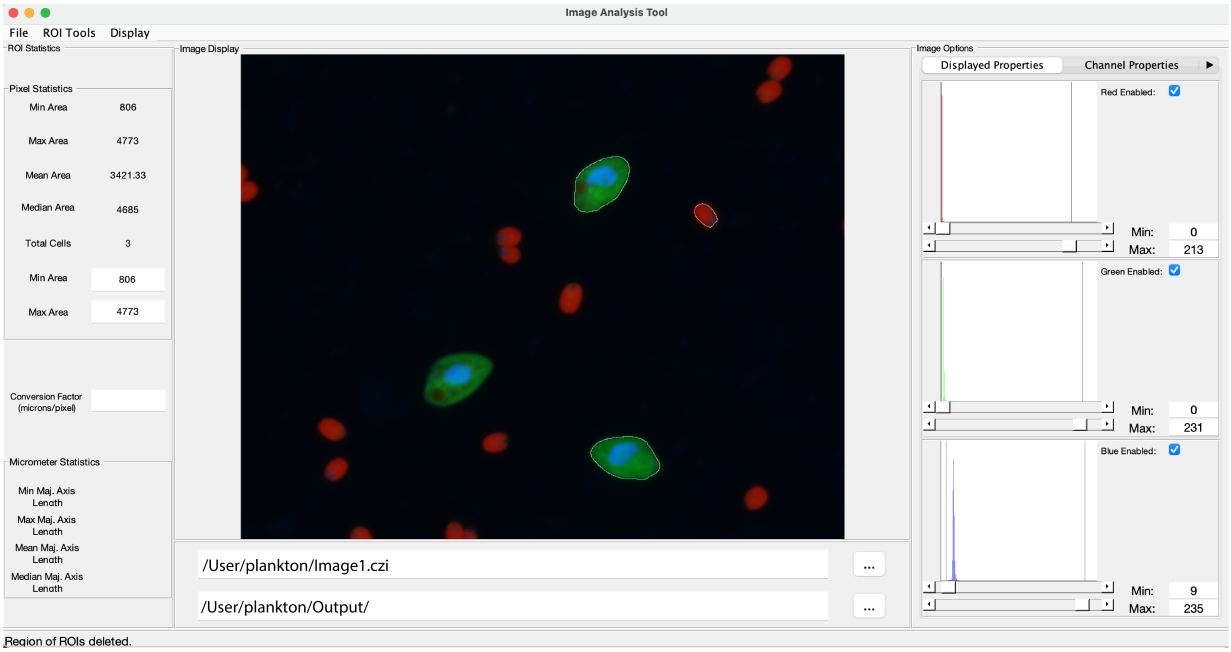



Figure 34: Delete Region of ROIs

### 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,

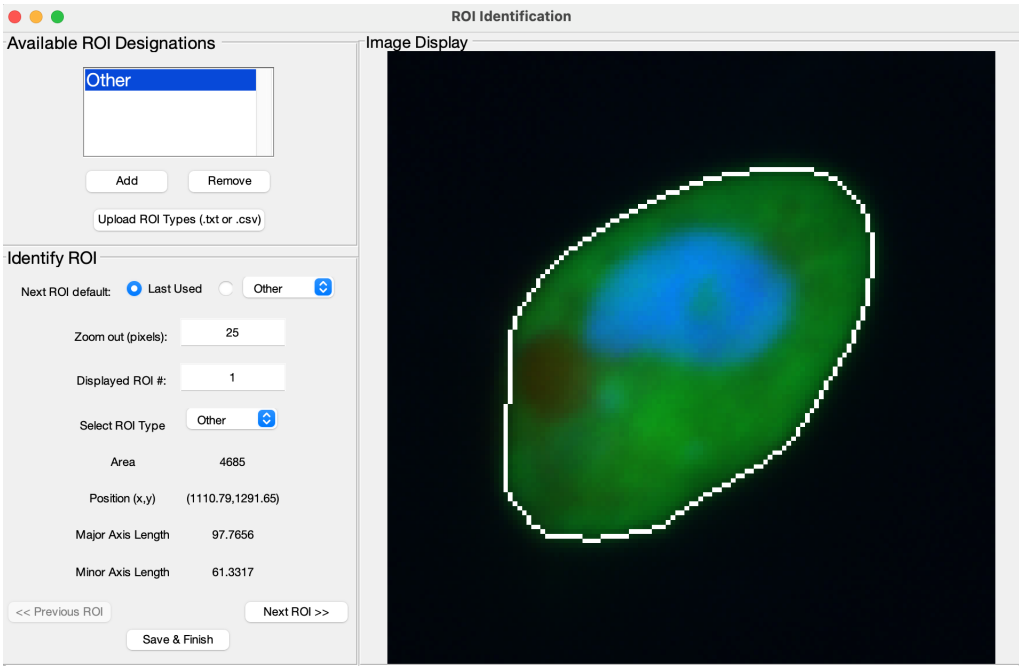



Figure 35: ROI Identification Tool

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 with 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.

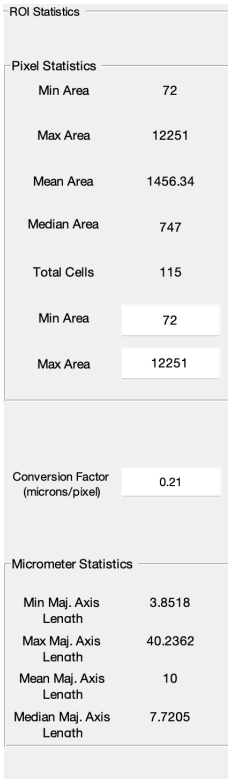



Figure 37: ROI Statistics Panel

## 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.

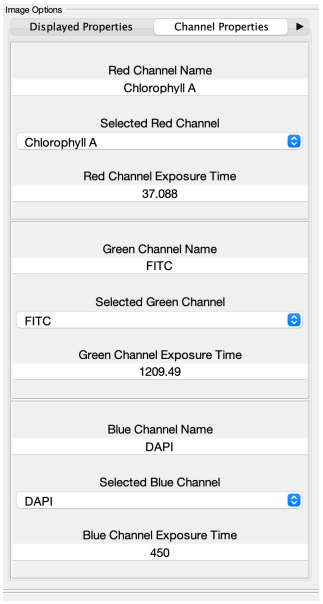



Figure 38: Channel Properties Tab

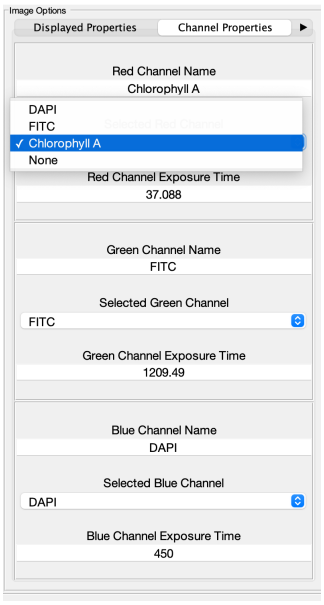



Figure 39: Example Channel Swap Dropdown

### 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'.

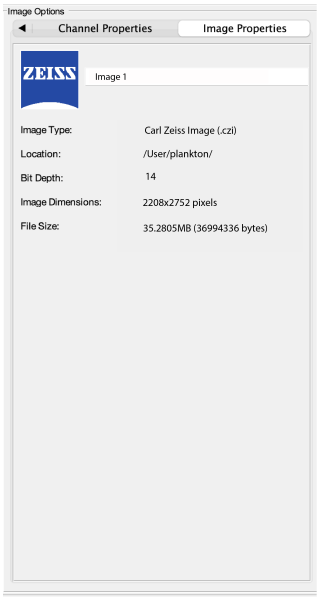



Figure 40: Image Properties Tab

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