

ViA User Guide

Alexis Pasulka, Jonathan Hood

Cal Poly San Luis Obispo ©2022

Introduction

The Viral Image Analysis (ViA) program was designed specifically for analyzing epifluorescent images of viral particles. The program can work with single channel images (e.g., DNA-stained viral particles) or two-channel images (e.g., BONCAT-tagged or FISHed viral particles with a DNA counter stain). The program is compatible with grayscale images in a .tiff format, as well as those in a .czi format (i.e., Zeiss images). It can load any number of channels for a given image, though only two may be analyzed at any one time. After analysis, the statistical data can be exported as a .txt, .csv, or .xlsx file.

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I System Requirements

The ViA 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 R2021b or later.
2. Platform-specific ViA program files.

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

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

2 Installation Procedure

2.1 MATLAB Program Version

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

2.2 Executable Program Version

Download the ViA executable appropriate to your system (Mac or Windows). For Windows this is a .exe file, and for Mac this is a .app file. Once you've downloaded the executable installer, you will need to navigate its location and run 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 and you are good to go. (*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 install, 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 ViA installer from opening due to developer permission issues. You may receive a pop-up message that says "macOS can not verify the developer of ViA 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 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*

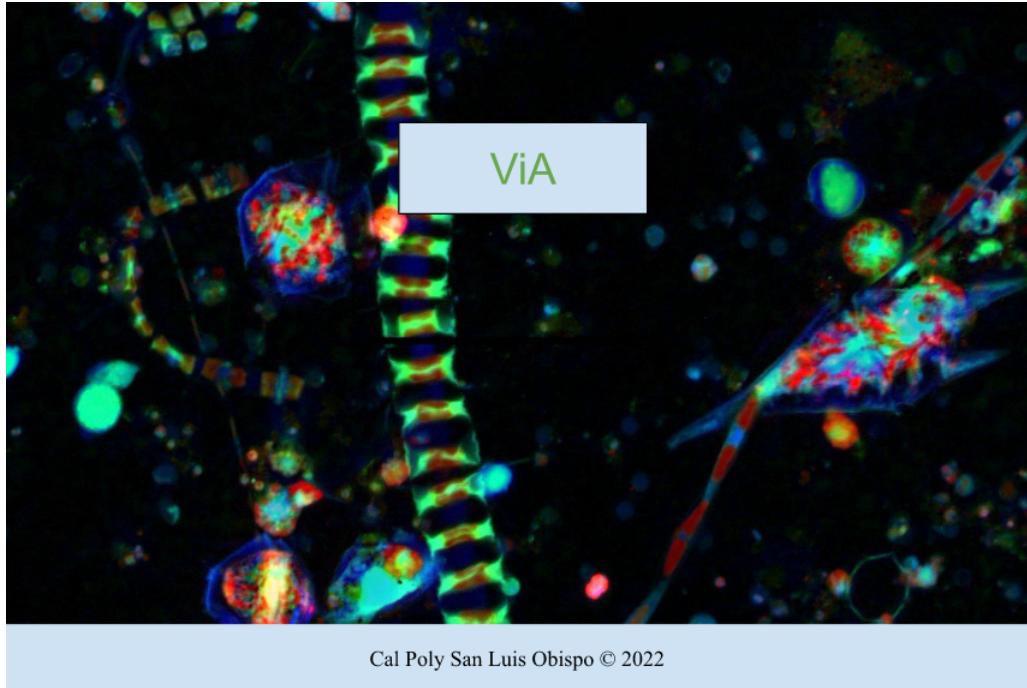


Figure 1: Windows ViA Splash Screen, 2022 edition

click “Open Anyway” next to the message “ViA 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 a .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; 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 'virus_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 'virus_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.

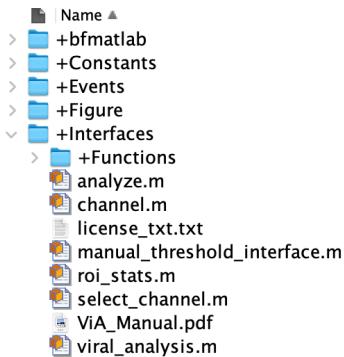


Figure 2: Working Directory

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

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 three primary sections; the center-left panel, which displays the loaded image(s); the center-bottom panel, which holds the filepaths to the loaded image and to the selected output directory; and the rightmost panel, which holds the analysis steps and options.

All tabs except for 'Raw Image' and 'Data Display' are initially disabled. The remaining tabs will become enabled as you work through the step by step procedure. To move through each image processing step, the user can click the 'Next Step' button located in the lower right corner of the 'Viral Analysis Procedure' panel. The user can also switch between any enabled step by simply selecting the desired tab.

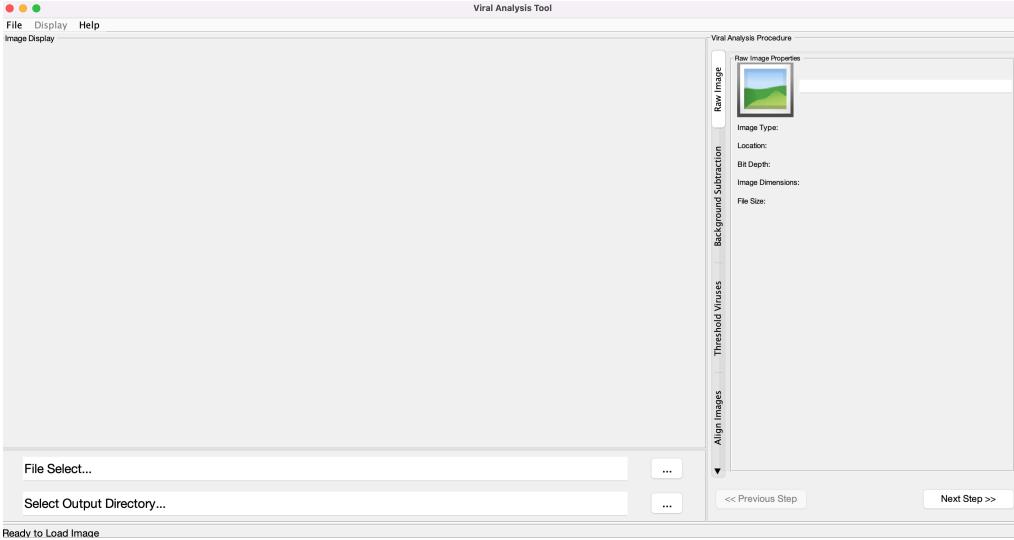


Figure 3: Initial Viral Analysis Interface

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).

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

A small selection dialog will pop up, allowing you to assign loaded channels to the two available comparison options visualized in Figure 4.



Figure 4: Channel Select Pop-up

The DNA channel label represents the primary channel and the LABLED channel option represents the secondary channel – for example BONCAT-tagged viral particles or FISHed viral particles. Throughout the manual, these two image channels are referred to as DNA and LABLED.

Once loaded, image information is displayed in the 'Raw Image' tab of the 'Viral Analysis Procedure' panel on the rightmost side. While editing, you can view the original, unedited images at any time by returning to the 'Raw Image' tab.

The interface will look different if one channel is loaded (Figure 5) vs. if two channels are loaded (Figure 6).

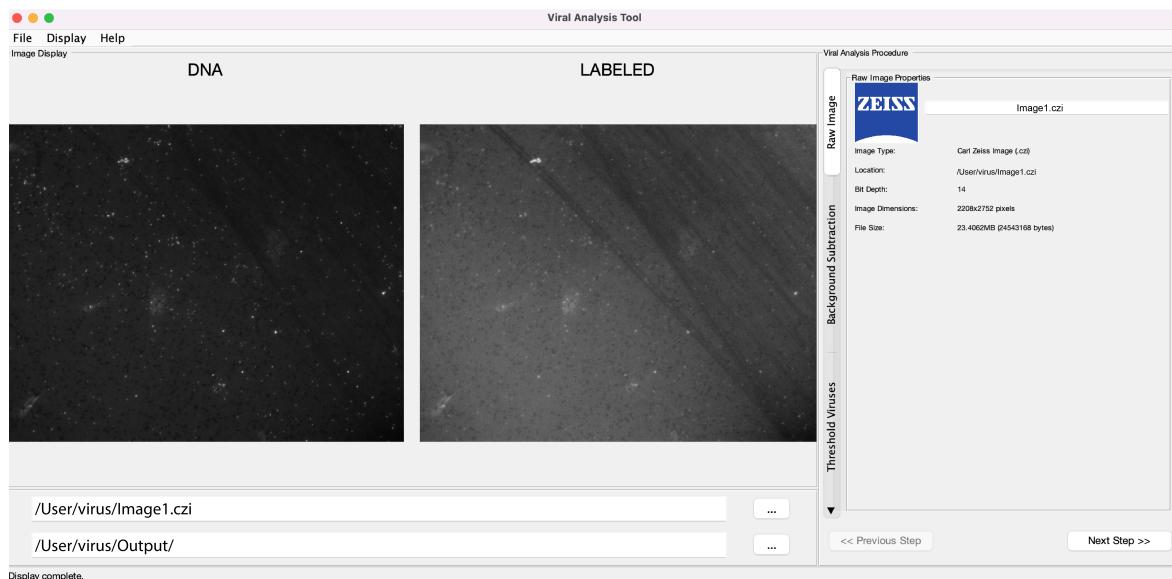


Figure 5: Initial Interface with Two Loaded Channels

****WARNING****: There is currently an error in Mac OS Catalina 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.

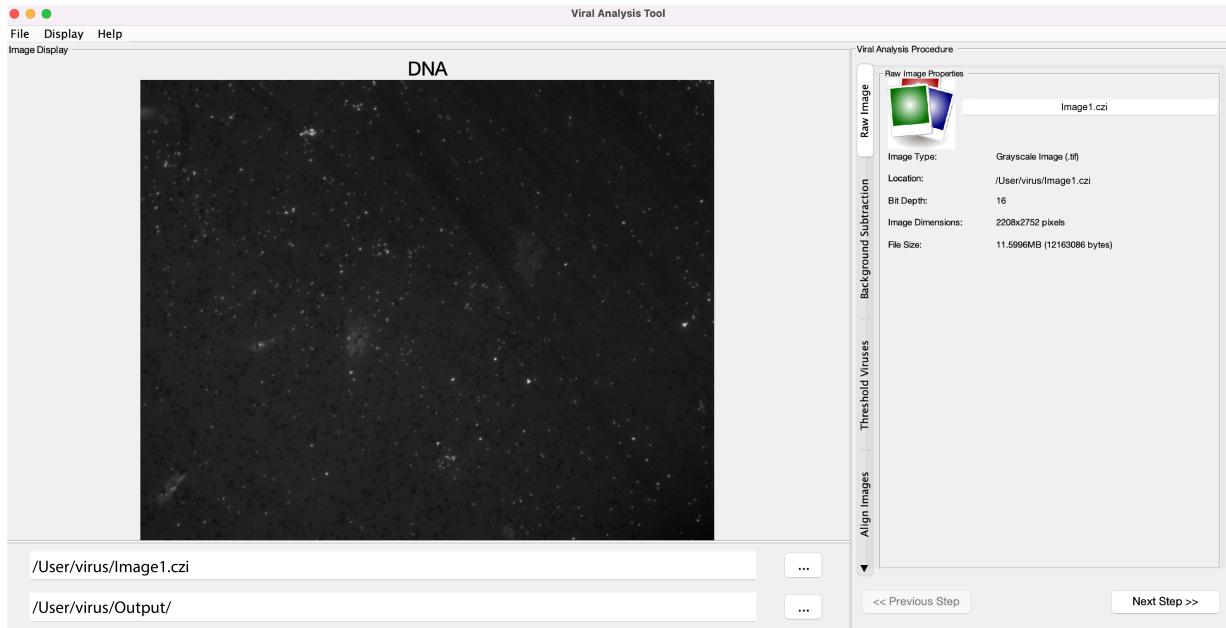


Figure 6: Initial Interface with One Channel Loaded

3.3 Changing Output Directory

The output directory is where all saved data will be placed. You can either enter the output directory in the editable text box as shown in Figure 7, 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 came from.



Figure 7: File-Directory Selection Snapshot

3.4 Background Subtraction

The first step in the image processing procedure is the background subtraction of the image(s). This aims to enhance the signal to noise ratio to detect and visualize viral particles. This tab will be enabled once you select 'Next Step' while in the 'Raw Image' tab. The program uses a rolling ball algorithm employing a disc configuration (default disc size = 10 pixels) and is applied automatically when the step is first activated. You may adjust the disc size manually as desired for both the DNA and LABELED images separately. An example background subtraction step display is shown in Figure 8.

Once the background has been removed to your satisfaction, hit 'Next Step' to enable the 'Threshold Viruses' tab and begin thresholding.

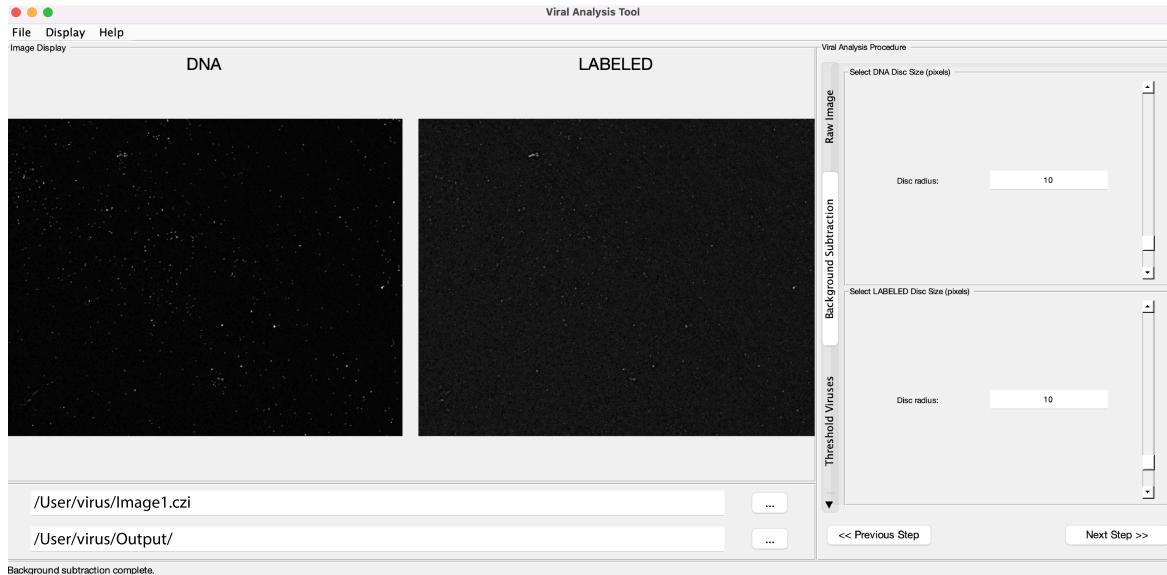


Figure 8: Example Background Subtraction Step Display

3.5 Threshold Viruses

The second step in image processing is a thresholding step (Figure 9). Upon first enabling this tab, the program will attempt to threshold the background subtracted image automatically utilizing Otsu's Method via MATLAB's `graythresh`. This converts the grayscale image to a binary image. If the threshold level for either image is too low to be detected, the threshold level for the other image is used. If both levels are too low, a default level of 0.01 is used. Thresholding levels will vary widely image to image, and thus manual adjustment is integral to successful image analysis.

Once the threshold has been set to your satisfaction, hit 'Next Step' to enable the subsequent tab. If two images were loaded, the 'Align Images' tab will be enabled. If only one image was loaded, this tab will not be enabled and the user will instead proceed directly to the removal of pixel artifacts. See section 3.7 for details.

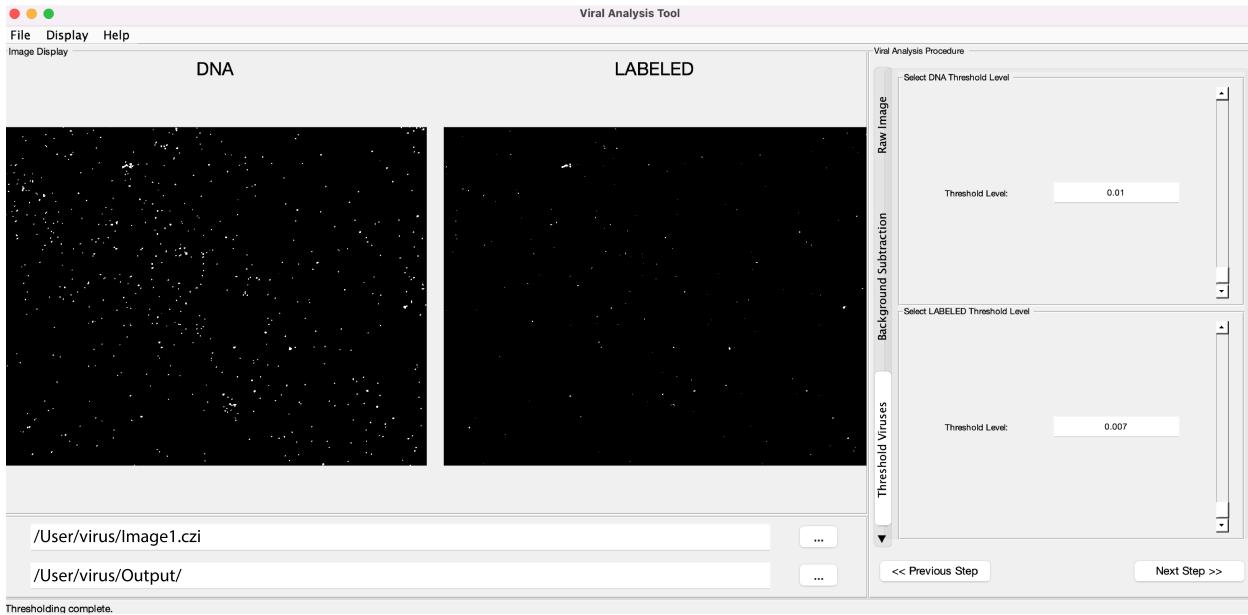


Figure 9: Example Thresholding Step Display

3.6 Align Images

In a one channel image analysis, this tab will stay disabled and be skipped. If two images were loaded, the third step is aligning the Labeled channel image to the DNA channel image, as shown in Figure 10.

The only editable feature on this tab is the maximum step length, specified as a positive scalar (the default is 0.0625). The initial step length is the same as the maximum step length because the optimizer reduces the step size during convergence. If you set the maximum step length to a large value, the computation time decreases. However, the optimizer might fail to converge if you set maximum step length to an overly large value.

After alignment, information describing how much the Labeled image was shifted in the X- and Y- directions in pixels is provided at the bottom of the panel. Once the images are aligned, hit 'Next Step' to enable the 'Remove Pixel Artifacts' tab.

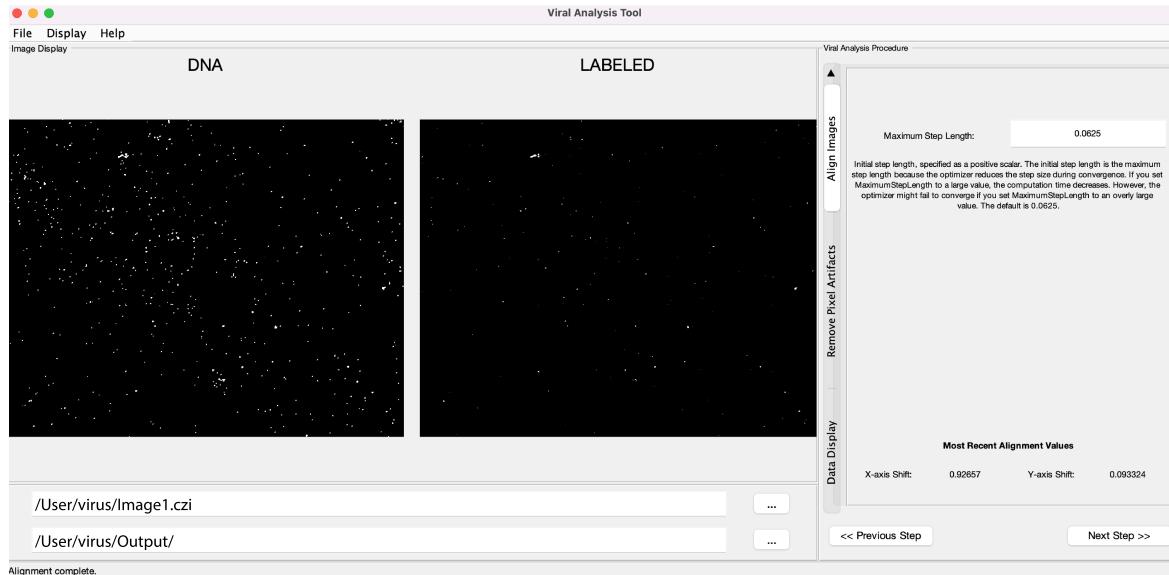


Figure 10: Example Alignment Step Display

3.7 Remove Pixel Artifacts

The fourth and final step is to remove any pixel artifacts from the image (Figure 11). This panel displays some general statistics of the DNA and the Labeled images including the minimum, maximum, and mean area of thresholded viral particles in square pixels as well as the total number of particles in each image ('Total ROIs'). The user can change the values in the min and max pixel text edit boxes and the program will filter out regions of interests smaller than the min and larger than the max and update the images and statistics accordingly.

There is also a conversion factor box here if the image micrometer to pixel ratio is known. If a value is entered here, the 'Micrometer Statistics Panel' is automatically filled with the same statistics as before except in micrometers instead of pixels.

Once the pixel artifacts have been removed, the general viral analysis procedure is complete. Any tab can be returned to and edited, and the 'Data Display' tab's histograms now have the most accurate information.

3.8 Data Display

The Data Display tab contains three histograms: the size of the particles, the Labeled:DNA background-subtracted fluorescence intensity ratios, and the Labeled:DNA intensity ratios (original data not background subtracted) (Figure 12). For each histogram, the number of bins as well as the X-axis range is editable. For the histograms displaying particle size, if pixels is selected in the drop-down menu, the data displayed is particle area (units = pixels²). However, if a micrometer conversion value was entered in the 'Remove Pixel Artifacts' tab, and micrometers is selected in the drop-down menu, the data displayed is 'Major Axis Length' (units = μm). When two images are loaded, the size data displayed in the histograms is from the DNA image. When only one image is loaded, the data reflect the size of particles in that one image.

Dual Zoom Option: There is also an option on this tab to enable/disable dual zoom. If dual zoom is

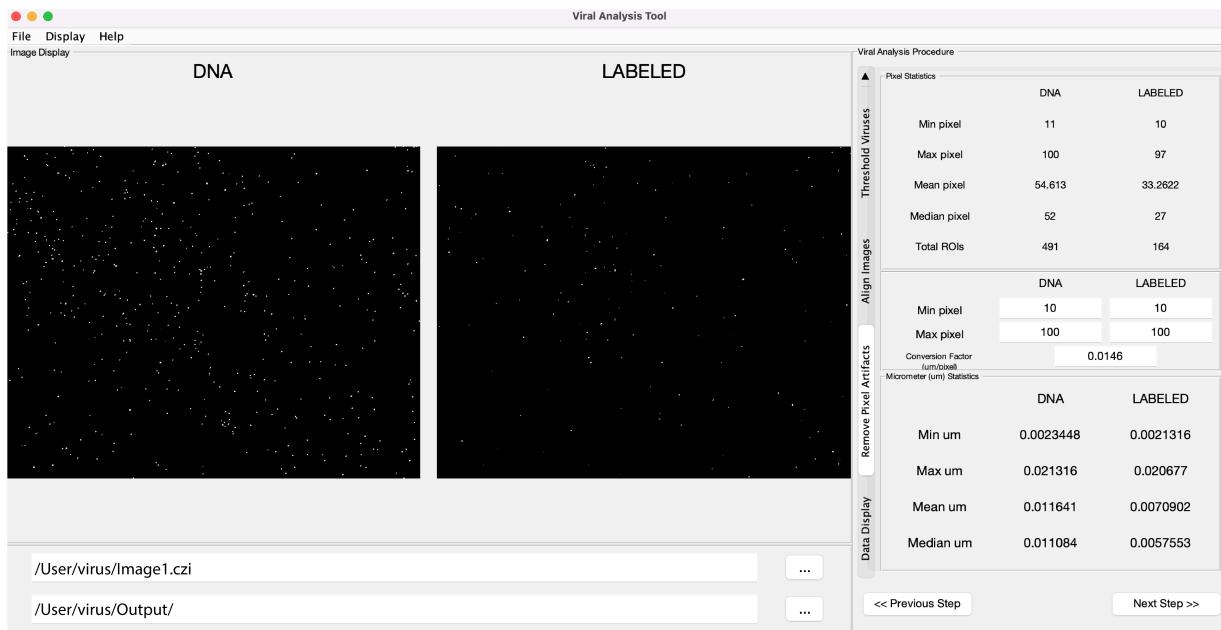


Figure 11: Example Artifact Removal Step Display

enabled, then when using the Zoom feature, the LABELED and DNA axes scales will synchronize and can be magnified and panned equivalently and simultaneously. With the feature off, only the axes the mouse is over will be affected by panning or magnification changes.

Display Centroid Option: While the identified particles are visible as white features on a black background, the centroid display option enables the user to display a red circle around the centroid of the cell (based on the DNA image particles if two images are loaded) to better visualize the particles (13). Since the same centroids are shown on both images, this can be particularly useful to quickly see which particles were selected based on the DNA image, but do not have a signal in LABELED image.

3.9 Saving Data, Histograms, & Images

Once you're done with image analysis, the user can save the data. There are four different save options, briefly listed in Table 1 below.

Once exported, you can work with the ROI data (in a .csv or .xlsx) in other programs (e.g., MATLAB, R, Excel).

4 Explanation of Menu Functions

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

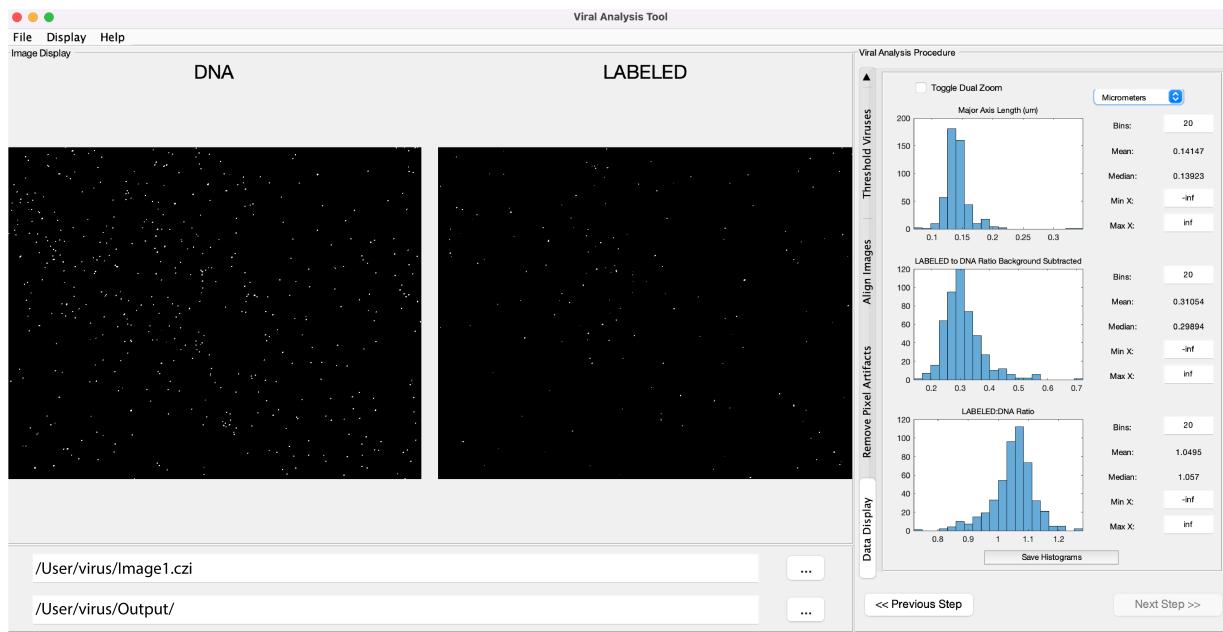


Figure 12: Example Data Display Panel

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 following the step-by-step procedure in the 'Viral Analysis Procedure' panel, employ one of the many 'Save' options (Table 1) to export the data.

4.1.1 Load Image

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

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

The default option during image selection is CZI, as shown in Figure 14.

The file extension can be changed to grayscale to display .tiff image options instead. With the grayscale file extension selected, the user can select any number of .tiff images. However, when 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, only two channels can be selected to fill the 'DNA' and 'LABELLED' slots at any given time.

If less than two images (or less than two channels within a CZI) are loaded, the user is presented with the option to assign the image or channel to either DNA or LABELLED slot. The slot not filled is

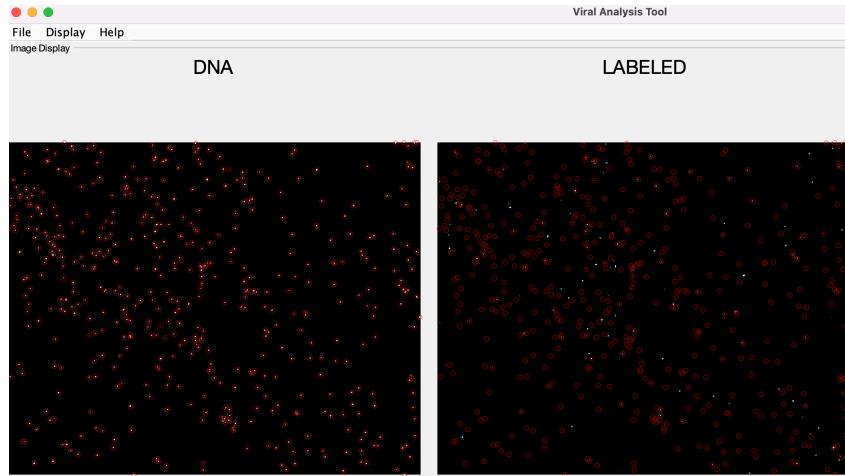


Figure 13: Example Display-Centroid Feature

Table 1: Available Save Options

Save Option	Description
Save Data	Exports ROI data based on current binary image masks. A full explanation of what data is exported is listed in Section 4.1.1.1.
Save Centroid Snapshot	Saves the two snapshots of the images created as a result of the 'Display Centroids' option under the 'Display' menu using the image filename as a prefix. See 4.2.1 for more information on the created centroid images.
Save Snapshot	Exports the current final binary image(s) under the image name. More detail for that operation can be seen in Section 4.1.1.3.
Save Histograms	Saves all of the displayed histograms as PNGs in the selected output directory with the image name as part of the image prefix.

then disabled throughout the viral analysis procedure. In addition, the 'Align Images' tab stays disabled and is skipped through the analysis. The histograms originally assigned to display ratio of intensities now instead display the fluorescence intensities of the individual cells in the image.

4.1.1.1 Save Data

The 'Save Data' option enables the user to save the statistical data from the analysis. The region of interest (ROI) data exports data based on the current final binary image masks. The ROI data will be exported to the selected output directory in the file format checked under 'Export ROI Data As...'.

Below is the information that gets saved for each image when exporting the data, where NAME indicates the name of each channel.

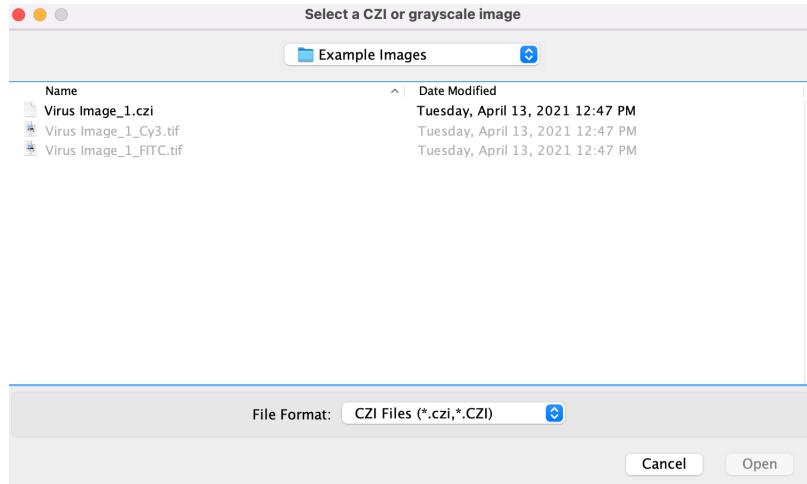


Figure 14: Example 'Load Image(s)' Dialog

NOTE: for single-image analysis, the centroid, area, and major axis length data is from the loaded image. For dual image analysis, those statistics are taken from the DNA image.

1. **image_name:** the name of the image itself, e.g. 'plankton_image.cz1'
2. **roi_num:** the individual ROI ID number, e.g. '1, 2, 3, 4, ...'
3. **area:** the area (actual number of pixels in the region) of the ROIs
4. **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.
5. **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.
6. **major_length:** the length of the major axis of the ROI, as approximated by an ellipse, in pixels.
7. **max_intensity_dna_raw:** the maximum intensity pixel of the raw DNA image within the ROI's area.
8. **max_intensity_dna_bs:** the maximum intensity pixel of the background-subtracted DNA image within the ROI's area.
9. **max_intensity_labeled_raw:** the maximum intensity pixel of the raw LABELED image within the ROI's area.
10. **max_intensity_labeled_bs:** the maximum intensity pixel of the background-subtracted DNA image within the ROI's area.

*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.*

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 pertinent data describing the analysis procedure; the total number of DNA and LABELED cells, the mean area of the DNA and LABELED cells in pixels, the DNA and

Labeled threshold levels employed, the DNA and labeled rolling ball disc size, the alignment translation values in the x- and y-, and the mean labeled:DNA raw and background-subtracted intensity ratio.

An example data output (Excel-type) is as displayed in Tables 2 and 3. 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.

Table 2: Example Output Data, Columns 1-7

image_name	roi_num	area	centroid_1	centroid_2	major_length	max_intensity_dna_raw
Virus_Example_1.tif	1	84	43.75	547.643	16.813	1971

Table 3: Example Output Data, Columns 8-10

max_intensity_dna_bs	max_intensity_labeled_raw	max_intensity_labeled_bs
1132	1414	121

4.1.1.2 Save Centroid Snapshot

Saves the two snapshots of the images created as a result of the 'Display Centroids' option under the 'Display' menu using the image filename as a prefix. See Section 4.2.1 for specific information on the creation of the centroid images.

4.1.1.3 Save Snapshot

'Save Snapshot' saves snapshots related to the current displayed setup. This enables the user to quickly share their current analysis. The snapshot ignores any centroids that may be displayed and any magnification of the images, and just saves PNGs of the final binary image.

4.1.2 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 4.1.1.1, 'Save Data'.

4.1.3 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. Ensure the data you wish to export has been saved, along with any histograms you'd like to keep images of.

4.2 Display Menu

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

4.2.1 Display Centroids

Selecting this option marks the centroid of each ROI in the DNA image and their corresponding locations in the LABELED image, as shown in Figure 13.

4.2.2 Zoom

'Zoom' enables the user to zoom in to 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 15). When Zoom mode is deactivated, this additional text will disappear. If dual Zoom mode has been toggled, the additional text in the figure title will instead read [DRAG-ZOOM: "on" (Synchronized XY)].

[DRAGZOOM : "on" (Normal)]: Viral Analysis Tool

Figure 15: Figure title when Zoom mode is on

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.
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.

Switching tabs and performing most actions will deactivate Zoom mode automatically. The current level of zoom will be maintained.

5 Licensing

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Version 3, 29 June 2007

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